

## 14-3-3 $\beta$ Binds to and Negatively Regulates the Tuberous Sclerosis Complex 2 (TSC2) Tumor Suppressor Gene Product, Tuberin\*

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**TSC2, or tuberin, is the product of the tuberous sclerosis tumor suppressor gene TSC2 and acts downstream of the phosphatidylinositol 3-kinase-Akt signaling pathway to negatively regulate cellular growth. One mechanism underlying its function is to assemble into a heterodimer with the TSC1 gene product TSC1, or hamartin, resulting in a reduction in phosphorylation, and hence activation, of the ribosomal subunit S6 kinase (S6K). We identified a novel interaction between TSC2 and 14-3-3 $\beta$ . We found that 14-3-3 $\beta$  does not interfere with TSC1-TSC2 binding and can form a ternary complex with these two proteins. Association between 14-3-3 $\beta$  and TSC2 requires phosphorylation of TSC2 at a unique residue that is not a known Akt phosphorylation site. The overexpression of 14-3-3 $\beta$  compromises the ability of the TSC1-TSC2 complex to reduce S6K phosphorylation. The antagonistic activity of 14-3-3 $\beta$  toward TSC is dependent on the 14-3-3 $\beta$ -TSC2 interaction, since a mutant of TSC2 that is not recognized by 14-3-3 $\beta$  is refractory to 14-3-3 $\beta$ . We suggest that 14-3-3 proteins interact with the TSC1-TSC2 complex and negatively regulate the function of the TSC proteins.**

Tuberous sclerosis (TSC)<sup>1</sup> is an inheritable disorder in which the brain, kidneys, skin, heart, and other organs may be affected by tumor-like growths, or hamartomas, that can result

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<sup>1</sup> The abbreviations used are: TSC, tuberous sclerosis complex; mTOR, mammalian target of rapamycin; GST, glutathione S-transferase; HA, hamagglutinin; CIP, calf intestinal phosphatase; S6K, subunit S6 kinase.

in seizures, mental retardation, and autism (1, 2). TSC affects roughly 1 in 6,000 newborns and is caused by mutations in either one of two genes. The *TSC1* locus encodes a 130-kDa protein termed hamartin, or TSC1, and the *TSC2* locus encodes a 180-kDa protein termed tuberin, or TSC2. Studies of hamartomas and tumors from TSC patients, as well as those of rodent models, support the categorization of both gene products as tumor suppressor proteins.

The proteins TSC1 and TSC2 bind each other, and this association is compromised by many tumor-derived mutations found in either protein (3). Studies in *Drosophila melanogaster* identified the TSC genes (*dTSC1* and *dTSC2*) as important regulators of cell growth (4–6). Direct biochemical evidence linking the function of the TSC complex to cell growth control was recently provided by the finding that TSC1-TSC2 inhibits mTOR effectors and that phosphorylation of TSC2 by Akt suppresses TSC1-TSC2 activity (7–10). In an effort to elucidate the function and regulation of TSC proteins, we searched for cellular protein(s) that interact with TSC2 and have identified 14-3-3 as a novel TSC2-interacting protein. We show that 14-3-3 selectively interacts with phosphorylated TSC2 and interferes with the function of TSC1-TSC2. Our findings identify a novel regulation of the TSC complex and provide further insight into the mechanisms that control TSC activity in cell signaling.

### EXPERIMENTAL PROCEDURES

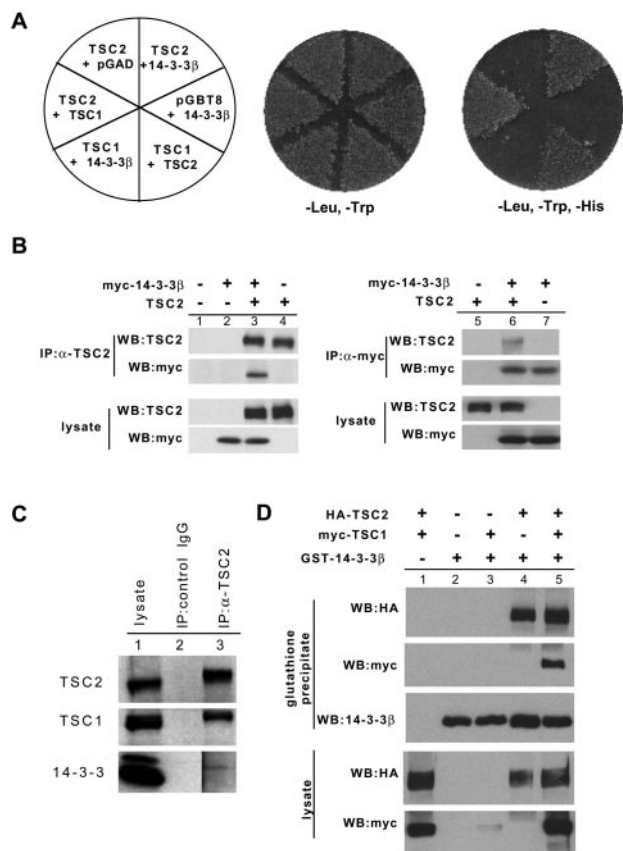
**Cell Culture, Yeast, Reagents, and Plasmids**—U2OS and HEK293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin, and streptomycin (Invitrogen). Insulin was purchased from Invitrogen and was administered at 350 nM. The HF7c strain of *Saccharomyces cerevisiae* was used in two-hybrid assays. cDNA encoding 14-3-3 $\beta$  was PCR amplified from the HeLa cDNA library and subcloned in-frame with either Myc-, HA-, or GST-epitope tags. Site-directed mutagenesis was performed by standard PCR techniques using the QuikChange kit (Stratagene) and verified by DNA sequencing.

**GST Fusion Protein Pull-down Assay**—GST or GST fusion proteins were purified with glutathione-Sepharose following induction with 0.1 mM isopropyl-1-thio- $\beta$ -D-galactopyranoside over 4 h in exponentially growing *Escherichia coli*, BL21(DE3), cultured at 30 °C. For HEK293T cell lysate, transfected cells were lysed in a Nonidet P-40 lysis buffer (15 mM Tris-HCl, pH 7.5, 0.15 M NaCl, 0.5% Nonidet P-40, 1 mM PMSF, 1 mM dithiothreitol, 2  $\mu$ g/ml aprotinin, 2  $\mu$ g/ml leupeptin, 10  $\mu$ g/ml trypsin inhibitor, and 150  $\mu$ g/ml benzamide) and 1 mg incubated at 30 °C for 1 h with or without 10 units of CIP and terminated with 10  $\mu$ M Na<sub>2</sub>VO<sub>3</sub>. Equal amounts of GST or GST fusion proteins (~20  $\mu$ g) were used to precipitate binding proteins from 250  $\mu$ g of treated lysate.

**Immunoprecipitation and Western Blotting**—Except where indicated, cells were lysed in Nonidet P-40 lysis buffer and cleared by centrifugation. To immunoprecipitate endogenous 14-3-3 $\beta$ -TSC2 complexes, 1.5 mg of total protein lysate was used; for all other immunoprecipitations, 300–500  $\mu$ g of cell lysate was used. Western blotting was performed with 50–100  $\mu$ g of protein extract separated by SDS-PAGE and transferred to nitrocellulose membrane. Polyclonal antibody to TSC2 was raised using purified fusion protein consisting of GST and a fragment of human TSC2 (residues 1283–1807) as an immunogen. Affinity-purified antibodies to Myc (clone 9E10, NeoMarkers), HA (clone 12CA5, Roche Molecular Biochemicals), TSC2 (C-20, Santa Cruz), 14-3-3 $\beta$  (K19, Santa Cruz), and phosphorylated Thr<sup>389</sup> of S6K (#9205, Cell Signaling Technologies) were purchased commercially. Quantitative analyses were performed with Scion Image software (www.scioncorp.com/).

### RESULTS AND DISCUSSION

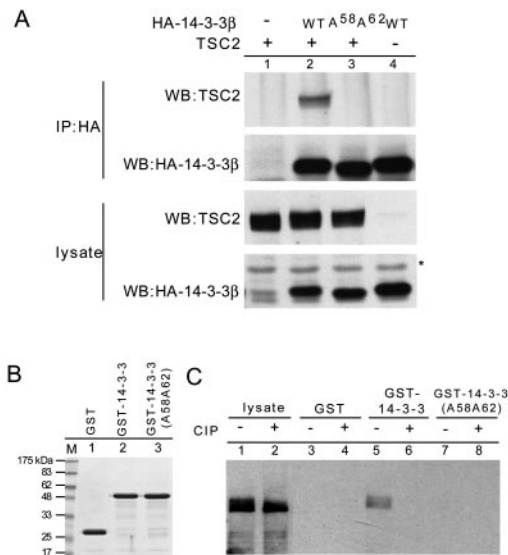
**Identification of 14-3-3 $\beta$  as a TSC2-interacting Protein**—In an attempt to uncover novel TSC2-interacting proteins we



**FIG. 1. 14-3-3β binds TSC2.** *A*, a directed yeast two-hybrid assay was performed by co-transforming yeast with bait construct (pGBT8) listed first and prey construct (pGAD) listed second, each with or without cDNA insert. Transformed cells were replica-plated to -Leu, -Trp plates and -Leu, -Trp, -His plates supplemented with 3-aminotriazole. *B*, HEK293T cells were transfected with plasmid encoding indicated proteins, and lysates were either immunoprecipitated (IP) first (upper two panels) or examined directly (lower two panels) by Western blotting with the indicated antibody (WB). *C*, lysate from exponentially growing HEK293T cells was immunoprecipitated with anti-Myc (control) or anti-TSC2 monoclonal antibodies and resolved by SDS-PAGE. Proteins were detected by immunoblotting with antibodies to TSC2, TSC1, and 14-3-3, and the Western blot shown is from a single exposure. *D*, experiment was performed as outlined in *B*.

screened  $3 \times 10^6$  independent clones derived from a HeLa cell cDNA library using full-length rat TSC2 as bait in a yeast two-hybrid assay. Among the 20 total positive colonies isolated in the screen were 11 independent clones of 14-3-3β and three of 14-3-3ζ. A directed yeast two-hybrid assay confirmed the binding of 14-3-3β to TSC2 but did not indicate an interaction between 14-3-3β and TSC1 (Fig. 1A). When TSC2 was ectopically expressed in HEK293T cells we were able to detect 14-3-3β in the anti-TSC2 immunoprecipitate (Fig. 1B). Reciprocally, TSC2 could also be detected in the 14-3-3β immunocomplex (Fig. 1B). An *in vivo* binding between endogenous TSC2 and 14-3-3 was detected in HEK293 cells (Fig. 1C). These results suggest that multiple isoforms of 14-3-3 can physically interact with the TSC2 gene product, tuberlin.

**14-3-3β Forms a Ternary Complex with TSC1 and TSC2 Proteins**—In contrast to tuberlin, TSC1 did not interact with 14-3-3β by yeast two-hybrid analysis (Fig. 1A). Since there is no obvious homologue to TSC1 in the genome of the host yeast, *S. cerevisiae*, these results indicate that TSC2 directly interacts with 14-3-3 and that this interaction is not dependent on the formation of a TSC1-TSC2 complex. Akt negatively regulates the TSC1-TSC2 complex by phosphorylating TSC2 and causing its disassembly from TSC1 (8, 9). We questioned whether bind-

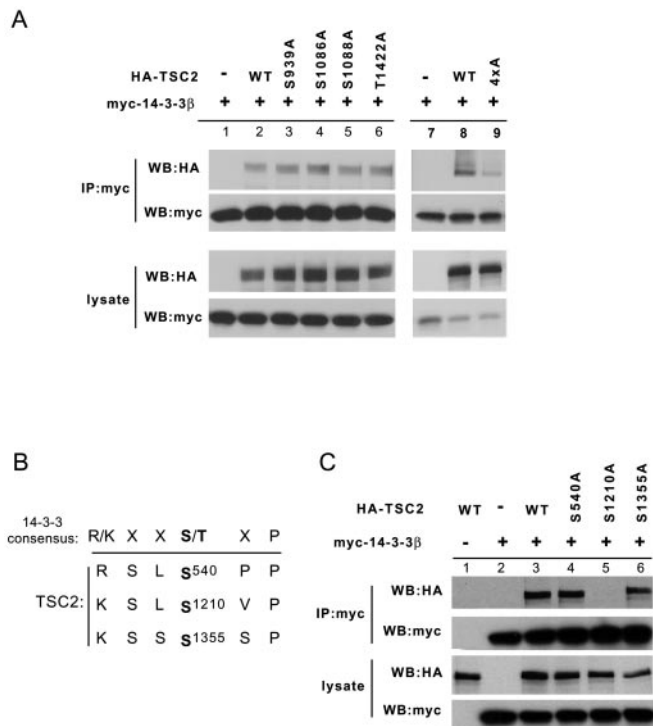


**FIG. 2. Binding of TSC2 by 14-3-3β requires phosphorylation of TSC2.** *A*, U2OS cells were transfected to express proteins as indicated and lysates were either immunoprecipitated (IP) with anti-HA antibody (upper two panels) or examined directly (lower two panels) by Western blotting with the indicated antibody (WB). \* indicates a nonspecific band. *B*, purified GST and GST fusion proteins were separated by SDS-PAGE followed by staining with Coomassie Brilliant Blue. Molecular mass standards are given to the left. *C*, lysate from HEK293T cells transfected with HA-TSC2 expression plasmid was incubated either with or without CIP as indicated and mixed with the GST or the GST fusion protein shown. Precipitated material was detected by Western blotting against the HA epitope. Equal amounts of each lysate (20% input) were included in lanes 1 and 2 to demonstrate even loading and CIP activity.

ing of 14-3-3β to TSC2 might similarly affect the TSC1-TSC2 interaction. We co-transfected plasmid encoding a GST-14-3-3β fusion protein into cells expressing Myc-TSC1 and HA-TSC2, either individually or in combination. GST-14-3-3β was precipitated from the lysate by addition of glutathione-agarose beads, and binding of TSC1 and TSC2 was examined by immunoblotting. Fig. 10 shows that GST-14-3-3β efficiently precipitated TSC2 (lane 4), but not TSC1 (lane 3). When overexpressed alone, the TSC1 protein presents at a very low level in the Nonidet P-40 lysate (lane 3), presumably due to its association into an insoluble fraction (11) and its rapid degradation when not complexed with TSC2.<sup>2</sup> When expressed with both TSC1 and TSC2, however, 14-3-3β was able to complex with TSC1 (Fig. 1D). Prolonged film exposure increased the signal of TSC1 (lane 3), but did not reveal any association of TSC1 with GST-14-3-3β (data not shown). These data indicate that binding of 14-3-3β with TSC2 does not impair TSC1-TSC2 association and that 14-3-3β can form a ternary complex with both proteins.

**14-3-3β Binds a Phosphorylated Form of Tuberlin**—Members of the 14-3-3 family of proteins typically interact with phosphoproteins, recognizing the consensus binding sequence RSXpS/TXP where pS/T represents a phosphorylated serine or threonine residue (12). Structural analyses have pinpointed a cluster of positively charged amino acids that are highly conserved among 14-3-3 proteins and that lay within an amphipathic groove on the surface of the protein (13, 14). These residues coordinate the phosphoamino acid located on the 14-3-3 binding partner, and their mutation completely disrupts the binding of 14-3-3 proteins to their specific interacting polypeptide (15–17). To determine whether these residues are also critical to the binding of TSC2 by 14-3-3β, we replaced

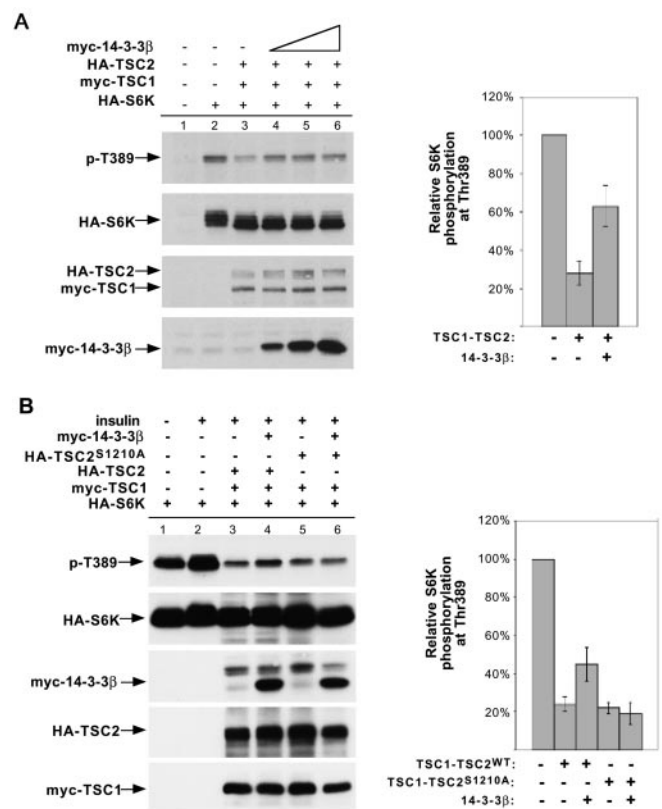
<sup>2</sup> S. Zacharek and Y. Xiong, unpublished observation.



**FIG. 3. Serine 1210 of TSC2 is necessary for 14-3-3 $\beta$  docking.** A, HEK293T cells were transfected with pcDNA3-Myc-14-3-3 $\beta$  and plasmid encoding the indicated mutant or wild-type form of HA-TSC2. Cells were lysed, immunoprecipitated (IP) with anti-Myc antibody, and immunoprecipitates examined by Western blot (WB) (upper two panels), and total cell lysate was analyzed in parallel (lower two panels). B, schematic diagram using single amino acid letter code to show consensus binding site for 14-3-3 proteins and putative 14-3-3 binding sites in TSC2. Phosphorylated residues are indicated in *bold type*. C, experiment was performed as outlined in A.

lysines 58 and 62 with alanine (14-3-3 $\beta$ <sup>K58A,K62A</sup>) and asked whether this mutant 14-3-3 $\beta$  was capable of associating with TSC2. No interaction between 14-3-3 $\beta$ <sup>K58A,K62A</sup> and TSC2 was detected (Fig. 2A), indicating that lysines 58 and 62 in 14-3-3 $\beta$  are essential for binding TSC2 and therefore that TSC2 may be recognized by 14-3-3 $\beta$  as a phosphoprotein. To further test this notion, we purified GST fusion proteins with both the wild-type 14-3-3 $\beta$  and the 14-3-3 $\beta$ <sup>K58A,K62A</sup> mutant (Fig. 2B). Equal amounts of these proteins were added to lysate from HEK293T cells expressing HA-TSC2 either left untreated or treated with CIP. Treatment with CIP caused the collapse of multiple otherwise broad TSC2 bands to a tight-packed species (Fig. 2C, lanes 1 and 2), confirming phosphorylation of TSC2. GST-14-3-3 $\beta$  was able to selectively precipitate slowly migrating phosphorylated forms of TSC2, but not the CIP-treated TSC2 (Fig. 2C, lanes 5 and 6). Neither GST alone nor GST-14-3-3 $\beta$ <sup>K58A,K62A</sup> was able to pull down TSC2 from the lysates. These findings demonstrate a requirement of TSC2 phosphorylation for binding to 14-3-3 $\beta$  and are in support of a direct interaction between the two polypeptides.

**Serine 1210 of TSC2 Is Required for 14-3-3 $\beta$  Binding**—The binding of phosphoproteins to 14-3-3s is often regulated by the serine/threonine protein kinases A, B/Akt, or C, since their phosphorylation consensus sites are similar to that recognized by 14-3-3s (18). Akt has been shown to directly phosphorylate TSC2 at multiple sites and thereby negatively regulate its activity (7–9). We questioned whether phosphorylation of TSC2 by Akt enabled its interaction with 14-3-3 $\beta$ . Mapping of Akt target sites in TSC2 by two-dimensional gel electrophoresis identified four residues phosphorylated by Akt: serine 939, serine 1086, serine 1088, and threonine 1422 (8). We tested



**FIG. 4. 14-3-3 $\beta$  decreases the ability of the TSC1-TSC2 complex to reduce S6K phosphorylation.** A, U2OS cells were transfected as indicated, and whole cell lysates were separated by SDS-PAGE followed by Western blotting. A phosphospecific antibody recognizing phosphothreonine 389 of S6K is shown as pT389. Five independent experiments were carried out as described, and S6K phosphorylation at Thr<sup>389</sup> as detected by Western blotting was quantified using Scion Image software. The lowest amounts of expressed 14-3-3 $\beta$  (lane 4) were used to calculate the *third bar*. B, HEK293T cells were transfected and serum-starved for 24 h prior to stimulation with 350 nM insulin for 30 min. Samples were analyzed as outlined in A, and quantification was performed on two independent experiments.

mutants of TSC2 in which each, or all (4 $\times$ A), of these residues was substituted by alanine to assess the role of Akt in regulating the TSC2–14-3-3 $\beta$  interaction. Strikingly, no single mutation had any effect on the ability of TSC2 to co-immunoprecipitate 14-3-3 $\beta$  (Fig. 3A). However, simultaneous mutation of all four Akt phosphorylation sites in TSC2 reduced its binding with 14-3-3 $\beta$  (4 $\times$ A mutant, lane 9). This suggested that 14-3-3 proteins bind TSC2 at a phosphoamino acid(s) distinct from those phosphorylated by Akt and that Akt-mediated phosphorylation, although not required, may influence the interaction of 14-3-3 with TSC2. With the aid of SCANSITE (scansite.mit.edu) we identified three putative 14-3-3 binding motifs within TSC2: serine 540, serine 1210, and serine 1355 (Fig. 3B). Binding analysis performed with the three individual TSC2 mutants revealed a requirement that Ser<sup>1210</sup> alone must remain intact in order for 14-3-3 $\beta$  to bind (Fig. 3C). Because Ser<sup>1210</sup> has not been identified as an Akt phosphorylation site (7–9), these results suggest that binding of 14-3-3 $\beta$  to TSC2 is dependent on a kinase(s) other than Akt.

**14-3-3 $\beta$  Antagonizes TSC Function**—The interaction of 14-3-3 proteins with various binding partners is known to have diverse biochemical consequences leading to various physiological effects (18). We were unable to detect altered subcellular localization of TSC2 following overexpression of 14-3-3 $\beta$  (data not shown). We were likewise unable to detect disruption of the TSC1-TSC2 protein complex nor an alteration in their stability

by overexpression of 14-3-3 $\beta$  (data not shown). Several recent reports have indicated that TSC1-TSC2 activity can be demonstrated by a reduction in S6K phosphorylation on threonine residue 389 (7–9). To determine whether there might be a functional consequence resulting from the interaction of 14-3-3 $\beta$  with TSC2, we assayed for the ability of TSC2 to inhibit phosphorylation, and hence activation, of S6K. Under normal growth conditions in U2OS cells S6K is phosphorylated on Thr<sup>389</sup>, and we detected an evident reduction in phosphorylation when TSC1 and TSC2 were co-expressed (Fig. 4A). Although increased expression of 14-3-3 $\beta$  did not alter the levels of either TSC1 or TSC2, the phosphorylation of Thr<sup>389</sup> on S6K was partially restored in the presence of 14-3-3 $\beta$  (Fig. 4A). In all experiments, however, we were unable to achieve a complete inhibition by 14-3-3 $\beta$  of the TSC1-TSC2 activity against S6K regardless of the cell line used, plasmid transfection ratios, or protein expression levels (data not shown). Nevertheless, in five independent experiments, co-expression of 14-3-3 $\beta$  was able to increase phosphorylation of Thr<sup>389</sup> in S6K an average of 2.3-fold over the levels observed in the presence of exogenous TSC1-TSC2 alone (Fig. 4A). This represents, on average, a 63% recovery of the S6K phosphothreonine 389 levels detectable in the absence of ectopically expressed TSC1-TSC2. The partial effect observed is consistent with the possibility that a portion of ectopically expressed TSC2 is refractory to 14-3-3 $\beta$  binding and, therefore, inhibition by 14-3-3 $\beta$ .

Ser<sup>1210</sup> in TSC2 is a probable site of phosphorylation by a yet unidentified kinase and is required for binding with 14-3-3 $\beta$  (Fig. 3C). To ensure that the ability of 14-3-3 $\beta$  to compromise the inhibition by TSC2 of S6K phosphorylation was due to the binding of 14-3-3 $\beta$  to TSC2 and not an indirect effect, we tested whether TSC2<sup>S1210A</sup> was also sensitive to regulation by 14-3-3 $\beta$ . The addition of insulin to serum-starved HEK293T cells caused an increase in phosphorylation of S6K at Thr<sup>389</sup> (Fig. 4B). Expression of TSC1 and TSC2 not only prevented the insulin-induced phosphorylation of S6K but caused the phosphothreonine 389 level to drop below that of serum-starved conditions (Fig. 4B, lanes 1–3). Although TSC2<sup>S1210A</sup> behaved like wild-type TSC2 to the extent that it could bind TSC1 (data not shown) and cause reduced phosphorylation of Thr<sup>389</sup> in S6K, the inhibitory activity of this mutant toward S6K was not affected by elevated amounts of 14-3-3 $\beta$ , whereas activity of the wild-type TSC2 was (Fig. 4B). These data are consistent with a regulatory scheme in which the binding of 14-3-3 $\beta$  to phosphoserine 1210 of TSC2 results in a diminished ability of the TSC1-TSC2 complex to negatively regulate the phosphorylation of S6K on Thr<sup>389</sup>.

Although mutant products of the *TSC1* and *TSC2* genes have long been implicated in tumorigenesis and dysregulation of cell growth, the mechanism underlying the function of these proteins in normal cell physiology has surfaced only recently (4–6). Genetic data indicating that the TSC1-TSC2 complex negatively regulates cell growth have been substantiated by biochemical studies, demonstrating inhibition of mTOR by TSC1-TSC2. The activity of the TSC complex to suppress mTOR is potent and can be inhibited by phosphorylation of TSC2 by Akt (7–10). Here we describe a distinct mechanism of regulating the growth-suppressive activity of the TSC complex. We have found that phosphorylated TSC2 is a ligand for 14-3-3 binding and that the functional consequence of this ternary

interaction is a decreased ability of TSC1-TSC2 to inhibit S6K phosphorylation, both basally and in response to stimulus. Importantly, this regulation of TSC by 14-3-3, unlike that by Akt phosphorylation, does not involve a dissociation of TSC1-TSC2.

Since Akt inactivates the TSC1-TSC2 complex via TSC2 phosphorylation, we were surprised to find that phosphorylation of TSC2 by Akt does not appear to provide the primary binding site for 14-3-3 $\beta$ . Rather, phosphorylation at serine 1210 of TSC2 by an unknown kinase is required for efficient binding of 14-3-3 $\beta$ . This implies that in addition to Akt, another kinase, and possibly a distinct cell growth-inducing signaling pathway, may converge at the inactivation of the TSC complex. We speculate that phosphorylation on serine 1210 of TSC2 is rate-limiting under our experimental conditions, since the inhibitory effect of increased 14-3-3 $\beta$  expression on TSC2 activity is saturable (Fig. 4A). With over 70 reported binding proteins to date, the cellular activities influenced by the 14-3-3 protein family is broad and diverse (18). Our results suggest that in mammalian cells 14-3-3 proteins may cooperate with mTOR to enhance cellular growth at least in part through the negative regulation of TSC1-TSC2. We provide evidence in support of a model in which direct, regulated binding by 14-3-3 to phosphorylated TSC2 does not disrupt the TSC1-TSC2 complex yet impairs its ability to function as a negative regulator of cell growth. The identification of the serine 1210 kinase and a determination of how 14-3-3 proteins restrain TSC activity by forming a ternary complex with TSC1-TSC2 will undoubtedly provide insight into the regulation and function of TSC in cell growth control.

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