Identification and Characterization of the Interaction between Tuberin and 14-3-3ζ*

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Tuberous sclerosis is caused by mutations to either the TSC1 or TSC2 tumor suppressor gene. The disease is characterized by a broad phenotypic spectrum that includes seizures, mental retardation, renal dysfunction, and dermatological abnormalities. TSC1 encodes a 130-kDa protein called hamartin, and TSC2 encodes a 200-kDa protein called tuberin. Although it has been shown that hamartin and tuberin form a complex and mediate phosphoinositide 3-kinase/Akt-dependent phosphorylation of the ribosomal protein S6, it is not yet clear how inactivation of either protein leads to tuberous sclerosis. Therefore, to obtain additional insight into tuberin and hamartin function, yeast two-hybrid screening experiments were performed to identify proteins that interact with tuberin. One of the proteins identified was 14-3-3ζ, a member of the 14-3-3 protein family. The interaction between tuberin and 14-3-3ζ was confirmed in vitro and by co-immunoprecipitation; multiple sites within tuberin for 14-3-3ζ binding were identified; and it was determined that 14-3-3ζ is associated with the tuberin-hamartin complex. Finally, it was shown that the tuberin/14-3-3ζ interaction is regulated by Akt-mediated phosphorylation of tuberin, providing insight into how tuberin may regulate phosphorylation of S6.

In humans, germ-line mutations to the TSC1 and TSC2 tumor suppressor genes cause the autosomal dominant disease tuberous sclerosis (TSC) (1, 2). The TSC1 gene encodes hamartin, a novel 130-kDa protein, whereas TSC2 encodes a 200-kDa protein called tuberin. Tuberin and hamartin interact to form a protein complex, and it has been suggested that inactivation of this complex leads to TSC (3). Indeed, pathogenic tuberin amino acid substitutions interfere with tuberin-hamartin complex formation (4, 5).

TSC is characterized by a variety of hamartomatous growths in different organs and tissues. The defects in cell proliferation, migration, and differentiation that these lesions display indicate that the TSC1 and TSC2 gene products participate in the control of cell growth and division (6). Overexpression of either tuberin or hamartin lengths G1 and inhibits cell proliferation, whereas G1 is shortened and tissues become hypertrophic when either gene is inactivated (7–12). Genetic studies in Drosophila provided the first evidence that tuberin and hamartin are involved in the phosphoinositide 3-kinase/protein kinase B (Akt) signal transduction pathway (11–13). More recently, it was shown that Akt interacts with and phosphorylates tuberin (14, 15) and that tuberin and hamartin regulate p70 S6 kinase activity and ribosomal protein S6 phosphorylation (16, 17). However, despite these exciting findings, it is not yet clear exactly how phosphoinositide 3-kinase/Akt signaling defects lead to the wide variety of lesions associated with TSC.

Previous work indicated that tuberin and hamartin interact within a large cytoplasmic complex, possibly containing other protein components (18). To identify the components of this complex and thereby obtain new clues toward the functions of tuberin and hamartin, yeast two-hybrid screening experiments were performed. An interaction between tuberin and 14-3-3ζ was identified.

14-3-3ζ belongs to a family of abundant 28–33-kDa acidic polypeptides (19). 14-3-3 proteins are broadly expressed and conserved in a wide range of eukaryotes. At least seven different isoforms have been identified in mammalian cells, and 14-3-3 proteins have also been shown to be essential for both budding and fission yeast viability (20). 14-3-3 proteins have been implicated in cell cycle control (21) and shown to interact with a wide range of signaling proteins (22). Many of these interactions are mediated through the binding of the 14-3-3 protein to two specific phosphoserine-containing sequences, RXxpSXP and RXXXpSXP, in the target protein (23). The involvement of 14-3-3ζ in phosphorylation-dependent regulation of signal transduction pathways made it an interesting candidate for a tuberin binding partner, and the interaction between tuberin and 14-3-3ζ was investigated in more detail.

The interaction between tuberin and 14-3-3ζ conformed to the pattern of binding of 14-3-3ζ to many other target proteins, and the interaction was shown to be representative of the binding between tuberin and the 14-3-3 protein family in general. The tuberin/14-3-3ζ interaction was shown to be dependent on Akt-mediated phosphorylation of tuberin, and evidence for multiple putative 14-3-3ζ-binding sites in tuberin was obtained.

EXPERIMENTAL PROCEDURES

DNA Constructs—The full-length TSC1 and TSC2 expression constructs have been described previously (3). Truncated TSC2 expression...
constructs encoding tuberin amino acids 1–607 (NruI truncation), 1–1099 (XmaI truncation), 1–252 plus 1536–1784 (SacI internal deletion), 607–1099 (NruI-XmaI fragment), and 1125–1784 (NruI-XmaI fragment) and the TSC2 R61IQ and R905Q variant expression constructs have also been described elsewhere (5). The TSC2 S540AA variant expression construct was obtained by site-directed mutagenesis of the original wild-type TSC2 expression construct using the Stratagene QuikChange site-directed mutagenesis kit. The yeast two-hybrid screening expression construct, a full-length TSC2 cDNA was cloned into the BamHII site of the pAS1 vector (Clontech). This construct was called pAS1-TSC2. The two full-length TSC2 variant constructs for the yeast two-hybrid experiments (pAS1-TSC2-R611IQ and pAS1-TSC2-R905Q) were obtained by site-directed mutagenesis of the pAS1-TSC2 construct.

For the transfection experiments, a full-length 14-3-3ζ cDNA, identified from yeast two-hybrid screening experiments, was cloned as a BamHI-BglII fragment into the BamHI cloning sites of the pcDNA3.1 and pcDNA3.1His mammalian expression vectors. For production of a glutathione S-transferase (GST)-14-3-3ζ recombinant fusion protein, the full-length 14-3-3ζ cDNA was cloned as an EcoRI fragment from the pcDNA3.1 expression construct into the pGEX-2T vector. All of the constructs were sequenced completely.

Constituting constructs in GST fusion proteins of the β, ε, γ, and η isoforms of 14-3-3 are kindly provided by Dr. J. Zhai (University of Pennsylvania, Philadelphia, PA), and the GST-Ral binding domain construct was provided by Dr. M. van Triest and Professor J. L. Bos (University of Utrecht, Utrecht, The Netherlands). Mammalian expression constructs encoding activated, wild-type, and dominant-negative Akt, p38, CREB, and Erk were purchased from Upstate Biotechnology, Inc.

Using a GST-14-3-3ζ full-length tuberin fusion protein as bait (pAS1-TSC2) was performed on a human adult brain cDNA library using a bait construct encoding full-length tuberin. From the 160 colonies that grew on selective medium, 115 were positive in a β-galactosidase selection assay, and 54 clones were isolated and sequenced. Of these, five independent clones encoded full-length 14-3-3ζ. The interactions between the full-length 14-3-3ζ clones and the tuberin bait construct were re-tested in the yeast two-hybrid assay. As shown in Table I, the interaction between 14-3-3ζ and tuberin was confirmed, and no interaction between 14-3-3ζ and either a bait construct encoding the Gal4 binding domain only or a bait construct encoding a fusion of the Gal4 binding domain with the hamartin coiled coil domain (pGBT-EE1b) (3) was detected. Next, the interaction between 14-3-3ζ and two different tuberin variants (R611IQ and R905Q) was investigated. Both the R611IQ and R905Q amino acid substitutions resulted from pathogenic TSC2 mutations. Previous work has demonstrated that the R611IQ substitution inhibits tuberin phosphorylation and disrupts the interaction between tuberin and hamartin, whereas the R905Q substitution does not (5). As shown in Table I, β-galactosidase activity was stimulated when the bait constructs pAS1-TSC2 and pAS1-TSC2-R905Q (encoding wild-type tuberin and the tuberin R905Q variant) were cotransformed with either pACT2-14-3-3ζ or pGAD-EE1a (encoding hamartin amino acids 334–1153). In contrast, β-galactosidase activity was not stimulated when the R611IQ variant was tested. The results from the yeast two-hybrid experiments were therefore consistent with the published data on the interaction between hamartin and different tuberin variants (5) and indicated that the tuberin/14-3-3ζ interaction was sensitive to pathogenic tuberin amino acid substitutions.

**Confirmation of Tuberin/14-3-3ζ Binding in Vitro**—To verify that the interaction between tuberin and 14-3-3ζ was specific, COS-1 cells were transfected with a TSC2 expression construct. After 48 h, the cells were lysed, and the cleared lysate was incubated with a bacterially expressed GST-14-3-3ζ fusion protein bound to glutathione-Sepharose beads. Tuberin retained by the GST-14-3-3ζ beads was detected by immunoblotting. As shown in Fig. 1A, tuberin bound to the GST-14-3-3ζ beads, but not to glutathione-Sepharose beads alone, glutathione-Sepharose beads containing GST, or glutathione-Sepharose beads containing another GST fusion protein (GST-Ral binding domain) (24).
The binding between wild-type tuberin and the GST-14-3-3ζ beads was compared with the binding of the tuberin R611Q and R905Q variants. COS-1 cells were transfected with expression constructs encoding the different tuberin variants, and the lysates were incubated with the GST-14-3-3ζ beads as described above. A third tuberin variant (S540A) was also tested (discussed below). As shown in Fig. 1B, the R611Q variant was retained by the GST-14-3-3ζ beads less efficiently than wild-type tuberin and the R905Q and S540A variants, consistent with the results from the yeast two-hybrid experiments.

**Interaction between 14-3-3ζ and the Tuberin-Hamartin Complex**—To investigate whether the tuberin/14-3-3ζ interaction affects the interaction between tuberin and hamartin, cleared lysates from COS-1 cells overexpressing tuberin, hamartin, or both proteins were incubated with the GST-14-3-3ζ beads. As shown in Fig. 2A, hamartin was retained by the GST-14-3-3ζ beads when tuberin was coexpressed, indicating either that the tuberin-hamartin complex was able to interact with the GST-14-3-3ζ beads or that hamartin could interact directly with 14-3-3ζ. In overexpressing cells, tuberin maintains hamartin in the soluble cytosolic fraction (18). Therefore, when a COS-1 cell lysate overexpressing only hamartin was tested in the binding assay, the assay was uninformative. Hamartin was not retained by the GST-14-3-3ζ beads because it was not present in the cleared cell lysate (Fig. 2A). Retention of hamartin by the GST-14-3-3ζ beads in the presence of tuberin indicated that the interaction between tuberin and 14-3-3ζ was compatible with the interaction between tuberin and hamartin.

A cleared lysate from HeLa cells expressing endogenous levels of tuberin and hamartin was tested in a GST-14-3-3ζ binding assay. As shown in Fig. 2B, both tuberin and hamartin were retained by the beads, whereas a control protein (FMRP) was not. The presence of the tuberin-hamartin complex in the HeLa lysate was demonstrated by the presence of tuberin in the hamartin immunoprecipitate. In Fig. 2B, the intensities of the signals for tuberin and hamartin were approximately equal in the immunoprecipitate fraction. In contrast, the tuberin signal in the lysate was more intense than the hamartin signal, indicating that only a proportion of the total tuberin in the cell was associated with hamartin. The weak hamartin signal in the GST-14-3-3ζ-bound fraction is consistent with the GST-14-3-3ζ beads binding both free tuberin and the tuberin-hamartin complex.

**In Vivo Interaction between Tuberin and 14-3-3ζ**—To investigate whether the interaction between tuberin and 14-3-3ζ occurs in vivo, co-immunoprecipitation experiments were performed.
Substitution of a single putative phosphorylated serine residue (Ser540) with alanine (RSLSPP to RSLAPP) did not prevent tuberin from binding to the GST-14-3-3ζ beads (Fig. 1B), isoforms of 14-3-3 (26). As shown in Fig. 4, both tuberin and hamartin were retained by all of the 14-3-3 isoforms tested, indicating that tuberin interacts with a domain common to the 14-3-3 protein family.

**Tuberin Contains Multiple Putative 14-3-3ζ-binding Sites**—Interaction between tuberin and the different 14-3-3 isoforms suggested that tuberin may contain a common binding motif recognized by all 14-3-3 proteins. Indeed, the 14-3-3 protein family binds many target proteins through two specific phosphoserine-containing motifs, RSxPxxP and RXRXpSXP, where pS represents phosphoserine and X is any amino acid (23). As illustrated in Fig. 5A, Scansite analysis of the amino acid sequence of tuberin identified eight potential 14-3-3-binding motifs (27): RSLSP (amino acids 537–542), KHSYTLPS (amino acids 599–605), RSTSLN (amino acids 936–941), RSISVS (amino acids 978–983), KSSSVP (amino acids 1251–1256), RSSSIVS (amino acids 1335–1340), KSSSSP (amino acids 1384–1389), and HRSRSNP (amino acids 1727–1732).

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consistent with the Scansite prediction that tuberin contains multiple 14-3-3-binding sites. The GST-14-3-3\(\beta\) binding assay was repeated using lysates from COS-1 cells transfected with expression constructs encoding different domains of tuberin. As shown in Fig. 5, all of the truncated tuberin proteins containing a putative 14-3-3-binding motif were retained by the GST-14-3-3\(\beta\) beads.

**Tuberin Phosphorylation Is Necessary for the Interaction between 14-3-3\(\beta\) and Tuberin**—To determine whether phosphorylation of tuberin at the predicted 14-3-3-binding motifs is necessary for the tuberin/14-3-3\(\beta\) interaction, the effect of phosphatase activity on the GST-14-3-3\(\beta\) binding assay was investigated. HeLa cell lysates were treated with protein phosphatase-1 before incubation with the GST-14-3-3\(\beta\) beads. The results of these experiments are shown in Fig. 6. After incubation for 1 h at 30 °C either with or without the addition of exogenous protein phosphatase-1, tuberin was no longer retained by the GST-14-3-3\(\beta\) beads. Proteolytic degradation of tuberin could be excluded because there was no reduction in the amount of tuberin in the lysate after incubation at 30 °C, and, as shown in Fig. 6B, the tuberin-hamartin complex was not disrupted by the 30 °C incubation. The lack of tuberin/14-3-3\(\beta\) binding was therefore consistent with dephosphorylation of tuberin by protein phosphatase-1, or another endogenous phosphatase, preventing binding to the GST-14-3-3\(\beta\) beads. Similar results were obtained with lysates of fibroblasts and transfected COS-1 cells (data not shown).

The binding assay was repeated in the presence of the phosphatase inhibitors Na\(_3\)VO\(_4\), NaF, and EDTA (28). As shown in Fig. 6C, retention of tuberin and hamartin by the GST-14-3-3\(\beta\) beads was improved by the addition of either 50 mM NaF or 50 mM EDTA to the cleared cell lysate, consistent with tuberin/14-3-3\(\beta\) binding being dependent on tuberin phosphorylation.

For comparison, the effects of the phosphatase inhibitors on tuberin/hamartin binding were also investigated. As shown in Fig. 6D, co-immunoprecipitation of the tuberin-hamartin complex was not affected by the addition of either 50 mM NaF or 50 mM EDTA, indicating that the tuberin/14-3-3\(\beta\) interaction is more sensitive to tuberin serine dephosphorylation compared with the tuberin/hamartin interaction.

The addition of 10 mM Na\(_3\)VO\(_4\) completely inhibited tuberin binding to the GST-14-3-3\(\beta\) beads. However, analysis of the GST-14-3-3\(\beta\) beads after the 10 mM Na\(_3\)VO\(_4\) treatment indicated that Na\(_3\)VO\(_4\) disrupted glutathione-Sepharose/GST binding, possibly due to glutathione oxidation (data not shown) (28). The tuberin-hamartin complex was also completely disrupted by the presence of 10 mM Na\(_3\)VO\(_4\). When 10 mM Na\(_3\)VO\(_4\) was added to the cell lysate, tuberin and hamartin could not be co-immunoprecipitated by antibodies specific for either tuberin or hamartin. However, direct immunoprecipitation of either protein alone was unaffected by the presence of 10 mM Na\(_3\)VO\(_4\). This indicated that 10 mM Na\(_3\)VO\(_4\) had a specific effect on the interaction between tuberin and hamartin, without affecting...
FIG. 6. Tuberin phosphorylation is necessary for binding to 14-3-3ζ. A, phosphatase activity prevents tuberin/14-3-3ζ binding. A cleared HeLa cell lysate was incubated at 30 °C for 60 min (t = 60) either with (+) or without (−) protein phosphatase-1 (PP1) before incubation with the GST-14-3-3ζ beads. The lysate was also incubated with the beads directly, without the 30 °C incubation (t = 0). Tuberin retained by the GST-14-3-3ζ beads was detected by immunoblotting. After the 30 °C incubation, tuberin was no longer retained by the GST-14-3-3ζ beads. B, effect of phosphatase activity on the tuberin-hamartin complex. A cleared HeLa cell lysate was incubated with or without protein phosphatase-1 prior to immunoprecipitation with antibodies specific for tuberin (Tuberin IP) or hamartin (Hamartin IP). The phosphatase treatment did not prevent co-immunoprecipitation of tuberin and hamartin, although a slight change in the mobility of tuberin was observed, consistent with previous reports (4). C, effects of phosphatase inhibitors on the interaction between tuberin and 14-3-3ζ. NaF (50 mM), Na3VO4 (10 mM), or EDTA (50 mM) was added to a cleared HeLa cell lysate prior to incubation with the GST-14-3-3ζ beads. Tuberin and hamartin retained by the beads were detected by immunoblotting. The retention of both proteins was increased by the addition of both NaF and EDTA to the lysate, even after incubation of the lysate at 30 °C. D, effects of phosphatase inhibitors on the tuberin-hamartin complex. NaF, Na3VO4, or EDTA was added to a HeLa cell lysate prior to immunoprecipitation with antibodies specific for tuberin or hamartin. Co-immunoprecipitation of tuberin as part of the tuberin-hamartin complex using antibodies specific for hamartin was not affected by the presence of 50 mM NaF or 50 mM EDTA. In contrast, 10 mM Na3VO4 completely prevented co-immunoprecipitation of tuberin, indicating that Na3VO4 disrupted the tuberin-hamartin complex. Immunoprecipitation of either tuberin or hamartin alone was not affected by the presence of 10 mM Na3VO4.
the interactions between the two proteins and their respective antibodies.

Akt-mediated Phosphorylation of Tuberin Regulates Tuberin/14-3-3ζ Binding—Recent studies have identified tuberin as a target of Akt-mediated phosphorylation (14, 15). To investigate whether the interaction between phosphorylated tuberin and 14-3-3ζ is affected by Akt activity, COS-1 cells were co-transfected with expression constructs encoding three different tuberin variants (wild-type, R611Q, and R905Q) and wild-type Akt, activated Akt, or a dominant-negative Akt variant. Forty-eight hours after transfection, the cleared cell lysates were incubated with the GST-14-3-3ζ beads. A, the dominant-negative Akt isoform reduced the binding of all three tuberin variants to the GST-14-3-3ζ beads. B, wild-type Akt and activated Akt reduced the mobility of wild-type tuberin and the R905Q variant on SDS-polyacrylamide gel, but did not affect the mobility of the R611Q variant.

Fig. 7. Akt-mediated phosphorylation regulates tuberin/14-3-3ζ binding. COS-1 cell lysates overexpressing wild-type Akt (left), a dominant-negative isoform (−), or a constitutively active isoform (+) together with wild-type tuberin (TSC2) or the R611Q or R905Q variant were incubated with the GST-14-3-3ζ beads. A, the dominant-negative Akt isoform reduced the binding of all three tuberin variants to the GST-14-3-3ζ beads. B, wild-type Akt and activated Akt reduced the mobility of wild-type tuberin and the R905Q variant on SDS-polyacrylamide gel, but did not affect the mobility of the R611Q variant.

Discussion

DISCUSSION

TSC is caused by the inactivation of either tuberin or hamartin. Recent research indicates that tuberin and hamartin are involved in the phosphoinositide 3-kinase/Akt signal transduction pathway (11–17) and that defects in phosphoinositide 3-kinase/Akt signaling are therefore responsible for at least some of the lesions associated with TSC.

Tuberin is phosphorylated by Akt (14); and in cells lacking either tuberin or hamartin, S6, one of the prime targets of phosphoinositide 3-kinase/Akt signaling, is constitutively phosphorylated (16, 17). It has been suggested that the tuberin-hamartin complex may integrate different signals controlling p70 S6 kinase activity and therefore provide the missing link between the phosphoinositide 3-kinase/Akt signal cascade and regulation of the p70 S6 kinase (14). However, it is not clear how the tuberin-hamartin complex achieves this. One possibility is that phosphorylation relieves tuberin-mediated inhibition of p70 S6 kinase (14).

To obtain additional insight into tuberin and hamartin function, the yeast two-hybrid system was employed to identify proteins that interact with tuberin and thereby regulate the functions of tuberin, hamartin, and the tuberin-hamartin complex. One of the proteins identified was 14-3-3ζ, a member of the 14-3-3 protein family. 14-3-3 proteins influence the functions of many different proteins and play an important role in multiple cell pathways, notably those involved in signal transduction. 14-3-3 proteins regulate protein activity, control cytoplasmic-nuclear protein shuttling, target proteins for degradation or dephosphorylation, and act as scaffolding molecules to couple different proteins together (19, 22). Therefore, 14-3-3ζ was a good candidate for a potential downstream effector of tuberin in the phosphoinositide 3-kinase/Akt signaling cascade. The direct interaction between 14-3-3ζ and tuberin identified in the yeast two-hybrid system was confirmed by in vitro binding experiments and by co-immunoprecipitation. The binding experiments indicated that tuberin is able to interact with multiple 14-3-3 isoforms and that tuberin contains multiple 14-3-3-binding sites, consistent with ScanSite predictions of eight potential consensus 14-3-3-binding motifs in the tuberin amino acid sequence. The presence of multiple 14-3-3-binding sites in tuberin is consistent with the hypothesis that the tuberin-hamartin complex may integrate several different input signals (14).

In many cases, target protein phosphorylation regulates the binding between 14-3-3 proteins and their targets (19, 22). Several pieces of evidence suggest that 14-3-3ζ interacts specifically with phosphorylated isoforms of tuberin. Tuberin is known to be phosphorylated at multiple amino acid residues (4, 14); and in this study, a tuberin mutant that is not phosphorylated correctly did not interact with 14-3-3ζ. In addition, the phosphatase inhibitors NaF and EDTA promoted tuberin/14-3-3ζ binding, whereas a dominant-negative isoform of Akt inhibited tuberin/14-3-3ζ binding.

The interaction between phosphorylated tuberin and 14-3-3ζ suggests several possible mechanisms whereby Akt-dependent phosphorylation of tuberin may prevent the inhibition of p70 S6 kinase activity. The consensus Akt substrate recognition motif (RXRXX(S/T)) is similar to the RXXpSXP and RXXpSXP consensus 14-3-3-binding motifs. Indeed, ScanSite analysis predicts three putative Akt substrate motifs in tuberin (Ser1335, Ser1338, and Ser1339) that are also potential 14-3-3-binding motifs. At least one of these sites (Ser1335) has been shown to be a primary site for Akt-dependent phosphorylation of tuberin (14). Therefore, following phosphorylation of tuberin by Akt, at Ser1335 for example, 14-3-3ζ may bind to and prevent the tuberin-mediated inhibition of p70 S6 kinase. 14-3-3ζ may achieve this relief of inhibition in one of several ways. 14-3-3ζ binding may inactivate tuberin directly or target tuberin for degradation (19). Alternatively, 14-3-3ζ may modify the interactions between tuberin and other binding partners or promote cytoplasmic-nuclear shuttling of tuberin to physically separate...
Tuberin and p70 S6 kinase. Indeed, phosphorylation-dependent translocation of tuberin to the nucleus has been reported (29). Both the GST-14-3-3ζ binding assay and the co-immunoprecipitation experiments indicated that the interaction between tuberin and 14-3-3ζ was compatible with tuberin/hamartin binding. However, a direct interaction between hamartin and 14-3-3ζ could not be excluded by either the binding assay or the co-immunoprecipitation experiments. A medium stringency Scansite analysis of the hamartin amino acid sequence identified two potential 14-3-3-binding motifs, RLITEP (amino acids 336–341) and RNKSES (amino acids 1097–1102). However, consistent with only hamartin being retained by the GST-14-3-3ζ beads as part of the tuberin-hamartin complex, no interaction between hamartin and 14-3-3ζ was detected in the yeast two-hybrid assay.

In contrast to the interaction between tuberin and 14-3-3ζ, co-immunoprecipitation of the tuberin-hamartin complex was not affected by the presence of phosphate inhibitors such as NaF and EDTA, indicating that the mechanism of tuberin/14-3-3ζ binding is distinct from that of tuberin/hamartin binding. Interestingly, co-immunoprecipitation of tuberin and hamartin was inhibited by Na2VO4, without affecting the direct immunoprecipitation of either protein, indicating that Na2VO4 disrupted the tuberin-hamartin complex. The vanadate ion may disrupt tuberin/hamartin binding by competing with phosphoryl transfer (28), suggesting that the tuberin/hamartin interaction may be influenced by the phosphorylation status of one or both proteins, consistent with recent results (15).

14-3-3ζ did not interact with a pathogenic tuberin variant containing a R611Q substitution in either the yeast two-hybrid assay or the GST-14-3-3ζ binding experiments. The R611Q substitution also inhibits formation of the tuberin-hamartin complex and alters tuberin phosphorylation (5). Therefore, although the tuberin/14-3-3ζ and tuberin/hamartin interactions appear to be distinct, a single amino acid substitution is sufficient to block both interactions. It is possible that the R611Q substitution may cause a major conformational change that prevents both tuberin/14-3-3ζ and tuberin/hamartin binding as well as the interactions with Akt and possibly other kinases. Alternatively, aberrant phosphorylation of the R611Q variant may inhibit both the tuberin/14-3-3ζ and tuberin/hamartin interactions. A more detailed investigation of the influence of TSC2 missense mutations on tuberin function is currently in progress.

In summary, an interaction between tuberin and 14-3-3ζ was detected using the yeast two-hybrid system and confirmed in vitro and by co-immunoprecipitation experiments. The interaction between 14-3-3ζ and tuberin was promoted by the inhibition of phosphatase activity and by Akt-mediated phosphorylation of tuberin, providing a link between tuberin/14-3-3ζ binding and a role for tuberin in the phosphoinositide 3-kinase/Akt signaling cascade.

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