THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 42, Issue of October 18, pp. 39417–39424, 2002

Printed in U.S.A.

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

Received for publication, May 16, 2002, and in revised form, August 7, 2002
Published, JBC Papers in Press, August 9, 2002, DOI 10.1074/jbc.M204802200

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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 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] (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 lengthens G_1 and inhibits cell proliferation, whereas G_1 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, RS*X*pS*X*P and R*XXX*pS*X*P, 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

* This work was supported by the Tuberous Sclerosis Alliance (United States of America), Noortman B. V. (Maastricht, The Netherlands), the Michelle Foundation, the Nederlandse Organisatie voor Wetenschappelijk Onderzoek, and the Nationaal Epilepsie Fonds (The Netherlands). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: TSC, tuberous sclerosis; GST, glutathione *S*-transferase; FMRP, fragile X mental retardation protein.

constructs encoding tuberin amino acids 1–607 (*Nru*I truncation), 1–1099 (*Xma*I truncation), 1–252 plus 1536–1784 (*Sac*I internal deletion), 607–1099 (*Nru*I-*Xma*I fragment), and 1125–1784 (*Nru*I-*Xma*I fragment) and the *TSC2* R611Q and R905Q variant expression constructs have also been described elsewhere (5). The *TSC2* S540A variant expression construct was obtained by site-directed mutagenesis of the original wild-type *TSC2* expression construct using the Stratagene QuikChange kit. For the yeast two-hybrid screening experiments, the full-length *TSC2* cDNA was cloned into the *Bam*HI 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*-R611Q 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 in the yeast two-hybrid screening experiments, was cloned as a *Bam*HI-*Bgl*II fragment into the *Bam*HI cloning sites of the pcDNA3.1 and pcDNA3.1/his 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 *Eco*RI fragment from the pcDNA3.1 expression construct into the pGEX-2T vector. All of the constructs were sequenced completely.

Constructs encoding GST fusion proteins of the β, ϵ, γ, η, and τ isoforms of 14-3-3 were 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 isoforms were purchased from Upstate Biotechnology, Inc.

Yeast Two-hybrid Screens and Experiments—A yeast two-hybrid screen using a Gal4-full-length tuberin fusion protein as bait (pAS1-*TSC2*) was performed on a human adult brain library cloned into the pACT2 vector following the recommendations of the supplier (Clontech). Colonies that grew on selective medium (Leu^-/His^-/Trp^-) were screened by a β-galactosidase colony lift assay. The cDNA inserts of the positive colonies were amplified by PCR using primers complementary to the pACT2 vector (5′-taccactacaatggatgatg-3′ and 5′-gttgaagtgaacttgcgggg-3′) and sequenced. Subsequently, the pACT2-cDNA constructs were isolated from the positive yeast clones as recommended by the supplier, transformed into super-competent *Escherichia coli* by electroporation, grown under selection, and re-isolated. The purified constructs were then retested against the original pAS1-*TSC2* bait construct. To ensure that the interactions were specific, the positive clones were also tested against a Gal4-hamartin bait construct (pGBT-EE1b) (3), a Gal4 bait construct lacking a cDNA insert (pAS1), and two tuberin variant constructs (pAS1-*TSC2*-R611Q and pAS1-*TSC2*-R905Q). These experiments were performed as described previously (3, 18).

Mammalian Cell Culture and Transfections—All cells were cultured in Dulbecco's modified Eagle's medium containing 5% fetal calf serum, penicillin, and streptomycin. For transfection experiments, COS-1 cells were seeded into 30-cm^2 culture dishes and grown overnight to a confluency of 50–70%. Expression constructs were transfected into the cells using LipofectAMINE Plus reagent as recommended by the manufacturer (Invitrogen). Forty-two hours after transfection, the cells were harvested and lysed in 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100 (buffer A) as described previously (18).

Protein Phosphatase Treatments—HeLa cells (80-cm^2 culture dishes) were lysed in protein phosphatase-1 lysis buffer (50 mM Tris-HCl (pH 7.0), 0.1 mM EDTA, 5 mM dithiothreitol, and 0.5% Triton X-100) for 10 min on ice, and the lysate was cleared by centrifugation at 10,000 × *g* for 10 min at 4 °C. Protein phosphatase-1 (10 units; New England Biolabs Inc.) and $MnCl_2$ (1 mM final concentration) were added to the lysate, which was subsequently incubated for 60 min at 30 °C. As a control, the lysate was incubated for 60 min at 30 °C without protein phosphatase-1 and in the presence of different phosphatase inhibitors (50 mM NaF, 10 mM Na_3VO_4, and 50 mM EDTA).

In Vitro Binding Experiments—For the GST-14-3-3 binding experiments, GST fusion protein expression was induced for 3 h in a 500-ml culture of *E. coli* BL21 by the addition of 1 mM isopropyl-β-D-thiogalactopyranoside. The bacterial pellet was resuspended in 20 ml of phosphate-buffered saline containing 0.5 mM dithiothreitol and sonicated thoroughly (10 × 30 s), and Triton X-100 was added to a final concentration of 1%. After gentle mixing for 30 min at 4 °C, the lysate was spun at 12,000 × *g* for 10 min at 4 °C. The induced GST fusion protein in the supernatant fraction was bound to 250 μl of prewashed glutathione-Sepharose beads for 60 min at 4 °C. The beads were washed extensively with phosphate-buffered saline to remove nonspecifically bound bacterial proteins, and the quantity and purity of the GST fusion protein bound to the glutathione-Sepharose beads were analyzed by SDS-PAGE.

Cleared HeLa or transfected COS-1 cell lysates (300–500 μl) were added to 20 μl of the washed GST-14-3-3 beads and mixed by gentle rotation for 1–2 h at 4 °C. The beads were recovered by centrifugation (2500 × *g*, 15 s, 4 °C), washed three times with lysis buffer, and resuspended in SDS-PAGE loading buffer prior to immunoblot analysis.

Immunoprecipitations—Human brain cortex (~50 mg) was suspended in 1 ml of buffer A containing 50 mM NaF and sonicated twice for 10 s. After centrifugation at 10,000 × *g* for 10 min at 4 °C, the supernatant was recovered and incubated with antibodies specific for 14-3-3ζ on ice for 60 min before the addition of 30 μl of 50% protein A-Sepharose suspension. After gentle rotation for 90 min at 4 °C, the immunoprecipitates were washed three times with 700 μl of lysis buffer before being analyzed by immunoblotting.

Antibodies—The tuberin- and hamartin-specific antibodies used in this study have been described previously (3). The FMRP-specific antibody was provided by Dr. F. Tamanini (Erasmus University, Rotterdam, The Netherlands), and the tau-specific antibody was provided by Dr. E. van Herpen (Erasmus University). 14-3-3ζ-specific monoclonal antibodies were purchased from Santa Cruz Biotechnology (sc-629 and sc-1019) and used at the manufacturer's recommended dilutions.

RESULTS

Tuberin and 14-3-3ζ Interact in Yeast Two-hybrid Experiments—Yeast two-hybrid screening experiments were performed on ~5×10^5 clones from the 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 retested 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 (R611Q and R905Q) was investigated. Both the R611Q and R905Q amino acid substitutions resulted from pathogenic *TSC2* mutations. Previous work has demonstrated that the R611Q 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 (encoding 14-3-3ζ) or pGAD-EE1a (encoding hamartin amino acids 334–1153). In contrast, β-galactosidase activity was not stimulated when the R611Q 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. 1*A*, 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).

TABLE I
Interaction between tuberin and 14–3-3ζ in the yeast two-hybrid system

GBD[a] bait		GAD prey		Interaction (β-galactosidase filter lift assay)
Construct	Fusion protein	Construct	Fusion protein	
pAS1-*TSC2*	GBD-tuberin	pACT2-14-3-3ζ	GAD-14-3-3ζ	+++
pAS1-*TSC2*	GBD-tuberin	pGAD-EE1a	GAD-hamartin-(334-1153)	+++
pAS1-*TSC2*	GBD-tuberin	pACT2	GAD	–
pAS1-*TSC2*-R611Q	GBD-tuberin R611Q	pACT2-14-3-3ζ	GAD-14-3-3ζ	–
pAS1-*TSC2*-R611Q	GBD-tuberin R611Q	pGAD-EE1a	GAD-hamartin-(334-1153)	–
pAS1-*TSC2*-R611Q	GBD-tuberin R611Q	pACT2	GAD	–
pAS1-*TSC2*-R905Q	GBD-tuberin R905Q	pACT2-14-3-3ζ	GAD-14-3-3ζ	+++
pAS1-*TSC2*-R905Q	GBD-tuberin R905Q	pGAD-EE1a	GAD-hamartin-(334-1153)	+++
pAS1-*TSC2*-R905Q	GBD-tuberin R905Q	pACT2	GAD	–
pGBT-EE1b	GBD-hamartin-(334-1153)	pACT2-14-3-3ζ	GAD-14-3-3ζ	–
pAS	GBD	pACT2-14-3-3ζ	GAD-14-3-3ζ	–

[a] GBD, Gal4 binding domain; GAD, Gal4 activating domain.

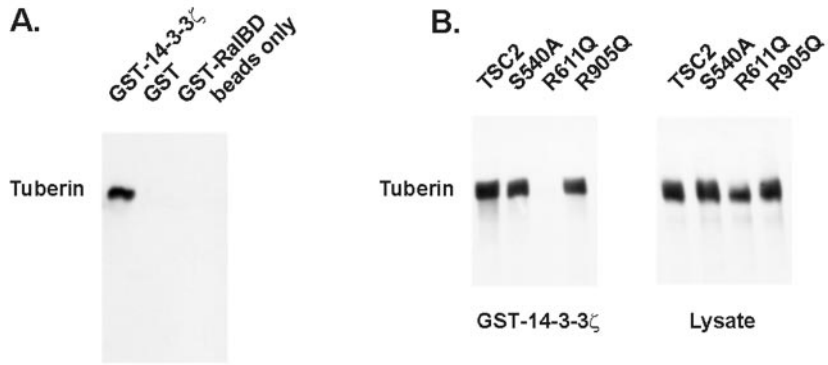

FIG. 1. **Tuberin binds specifically to a GST-14-3-3ζ fusion protein.** *A*, a lysate of COS-1 cells overexpressing wild-type tuberin was applied to glutathione-Sepharose beads alone or to glutathione-Sepharose beads prebound with the recombinant proteins GST-14-3-3ζ, GST, or GST-Ral binding protein (RalBD). Tuberin retained by the beads was detected by immunoblotting. Tuberin was retained only by the GST-14-3-3ζ beads. *B*, COS-1 cell lysates overexpressing wild-type tuberin (*TSC2*) or the S540A, R611Q, and R905Q variants were incubated with the GST-14-3-3ζ beads. Wild-type tuberin and the S540A and R905Q variants were retained by the beads more efficiently than the R611Q variant.

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. 1*B*, 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. 2*A*, 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. 2*A*). 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. 2*B*, 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. 2*B*, 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 per-

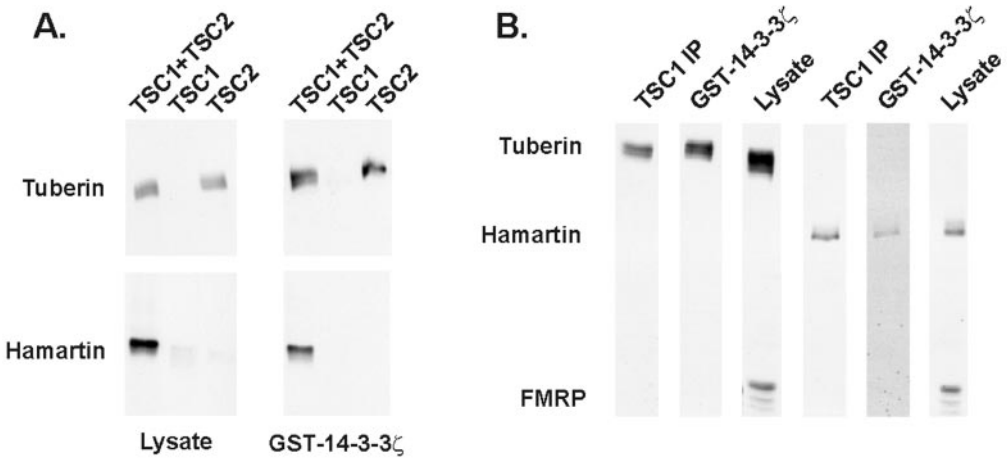

FIG. 2. **Interaction between GST-14-3-3ζ and the tuberin-hamartin complex.** *A*, COS-1 cell lysates overexpressing tuberin (*TSC2*), hamartin (*TSC1*), or both proteins (*TSC2+TSC1*) were incubated with the GST-14-3-3ζ beads. The amount of tuberin and hamartin in the lysates and retained by the beads was determined by immunoblotting. Tuberin was retained by the GST-14-3-3ζ beads irrespective of the presence of hamartin. In contrast, hamartin was detected only in the lysate and retained by the beads only when tuberin was coexpressed. *B*, a cleared HeLa cell lysate was incubated with the GST-14-3-3ζ beads and with antibodies specific for hamartin (*TSC1 IP*). The amounts of tuberin, hamartin, and FMRP in the lysate and in the hamartin immunoprecipitate and retained by the GST-14-3-3ζ beads were determined by immunoblotting. Tuberin was co-immunoprecipitated with hamartin, and both proteins were retained by the GST-14-3-3ζ beads. In contrast, FMRP was not co-immunoprecipitated with the tuberin-hamartin complex and was not retained by the GST-14-3-3ζ beads.

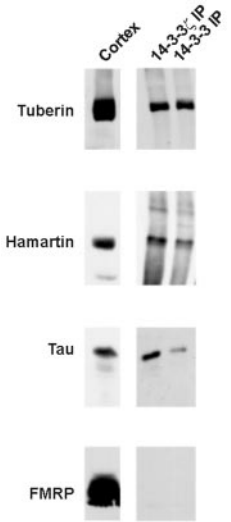

FIG. 3. **Co-immunoprecipitation of tuberin and 14-3-3ζ.** A cleared lysate was prepared from brain cortex. The expression of tuberin, hamartin, tau, and FMRP in the lysate is indicated (*Cortex*). Immunoprecipitation was performed with an antibody specific for 14-3-3ζ (*14-3-3ζ IP*) and with a broad specificity antibody against all 14-3-3 proteins (*14-3-3 IP*). Tuberin, hamartin, and tau were co-immunoprecipitated by both antibodies, whereas FMRP was not.

formed on lysates of HeLa cells as well as human brain cortex. Attempts to co-immunoprecipitate tuberin and 14-3-3ζ from HeLa cells were unsuccessful. However, as shown in Fig. 3, tuberin was co-immunoprecipitated from brain cortex by antibodies specific for 14-3-3ζ, indicating that tuberin and 14-3-3ζ associate *in vivo*. Consistent with the results of the GST-14-3-3ζ binding assay, hamartin was also detectable in the immunoprecipitate, whereas FMRP was not co-immunoprecipitated by the 14-3-3ζ-specific antibodies. Tau, another protein that is known to interact with 14-3-3ζ (25), was also detected in the immunoprecipitate.

Tuberin Interacts with Multiple 14-3-3 Isoforms—To investigate whether tuberin interacts only with 14-3-3ζ or also with additional members of the 14-3-3 protein family, a cleared HeLa cell lysate was incubated with glutathione-Sepharose beads containing GST fusion proteins of the β, ε, γ, η, and τ

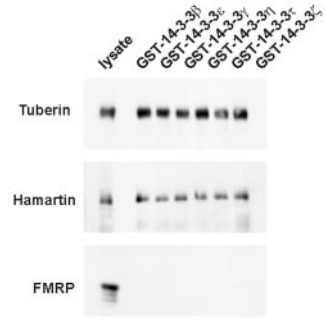

FIG. 4. **Interaction between tuberin and multiple 14-3-3 isoforms.** A HeLa cell lysate was incubated with glutathione-Sepharose beads containing GST-14-3-3β, GST-14-3-3ε, GST-14-3-3γ, GST-14-3-3η, GST-14-3-3τ, or GST-14-3-3ζ. Tuberin, hamartin, and FMRP in the lysate (*lysate*) and retained by the beads were detected by immunoblotting. Both tuberin and hamartin were retained by all of the 14-3-3 isoforms, whereas FMRP was not.

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, RS*X*pS*XP* and R*XXX*pS*XP*, where pS represents phosphoserine and *X* is any amino acid (23). As illustrated in Fig. 5*A*, Scansite analysis of the amino acid sequence of tuberin identified eight potential 14-3-3-binding motifs (27)[2]: RSLSPP (amino acids 537–542), KHSYTLP (amino acids 599–605), RSTSLN (amino acids 936–941), RSISVS (amino acids 978–983), KSLSVP (amino acids 1251–1256), RSSSVS (amino acids 1335–1340), KSSSSP (amino acids 1384–1389), and HSRSNP (amino acids 1727–1732).

Substitution of a single putative phosphorylated serine residue (Ser^{540}) with alanine (RSLSPP to RSLAPP) did not prevent tuberin from binding to the GST-14-3-3ζ beads (Fig. 1*B*),

[2] Available at Scansite.mit.edu.

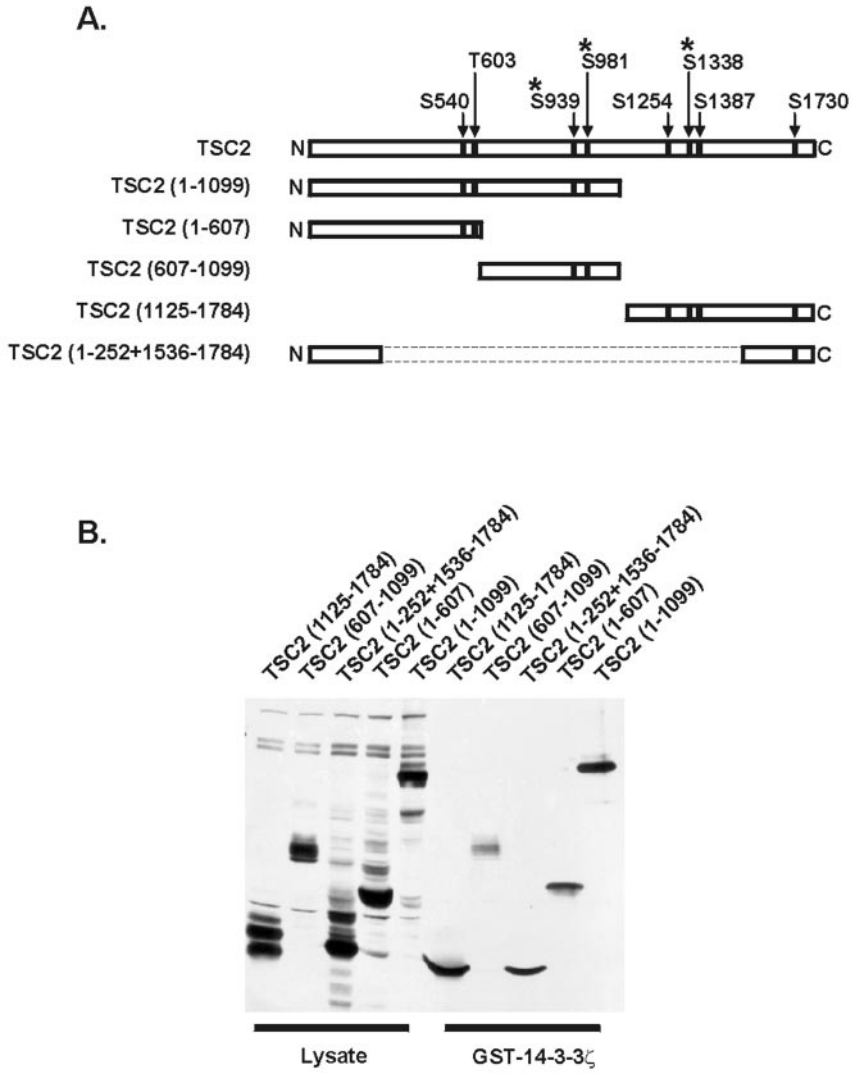

FIG. 5. **Interaction between 14-3-3ζ and multiple domains of tuberin.** *A*, shown is a schematic diagram indicating the extent of the truncated tuberin proteins and the relative positions of the putative 14-3-3-binding motifs predicted by Scansite. Akt phosphorylation sites are marked with *asterisks*. *N* and *C* represent the tuberin N and C termini, respectively; and the *dashed lines* indicate the extent of the in-frame deletion. *B*, COS-1 cell lysates overexpressing different truncated tuberin proteins containing an N-terminal polyhistidine epitope tag sequence were incubated with GST-14-3-3ζ beads. The amounts of the truncated tuberin proteins in the lysates and retained by the beads were determined by immunoblotting with an antibody against the epitope tag sequence. The domains encoded by the different truncated proteins are indicated.

consistent with the Scansite prediction that tuberin contains multiple 14-3-3-binding sites. The GST-14-3-3ζ 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-14-3-3ζ beads.

Tuberin Phosphorylation Is Necessary for the Interaction between 14-3-3ζ and Tuberin—To determine whether phosphorylation of tuberin at the predicted 14-3-3-binding motifs is necessary for the tuberin/14-3-3ζ interaction, the effect of phosphatase activity on the GST-14-3-3ζ binding assay was investigated. HeLa cell lysates were treated with protein phosphatase-1 before incubation with the GST-14-3-3ζ 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-14-3-3ζ 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. 6*B*, the tuberin-hamartin complex was not disrupted by the 30 °C incubation. The lack of tuberin/14-3-3ζ binding was therefore consistent with dephosphorylation of tuberin by protein phosphatase-1, or another endogenous phosphatase, preventing binding to the GST-14-3-3ζ 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_3VO_4, NaF, and EDTA (28). As shown in Fig. 6*C*, retention of tuberin and hamartin by the GST-14-3-3ζ beads was improved by the addition of either 50 mM NaF or 50 mM EDTA to the cleared cell lysate, consistent with tuberin/14-3-3ζ 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. 6*D*, 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/14-3-3ζ interaction is more sensitive to tuberin serine dephosphorylation compared with the tuberin/hamartin interaction.

The addition of 10 mM Na_3VO_4 completely inhibited tuberin binding to the GST-14-3-3ζ beads. However, analysis of the GST-14-3-3ζ beads after the 10 mM Na_3VO_4 treatment indicated that Na_3VO_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_3VO_4. When 10 mM Na_3VO_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_3VO_4. This indicated that 10 mM Na_3VO_4 had a specific effect on the interaction between tuberin and hamartin, without affecting

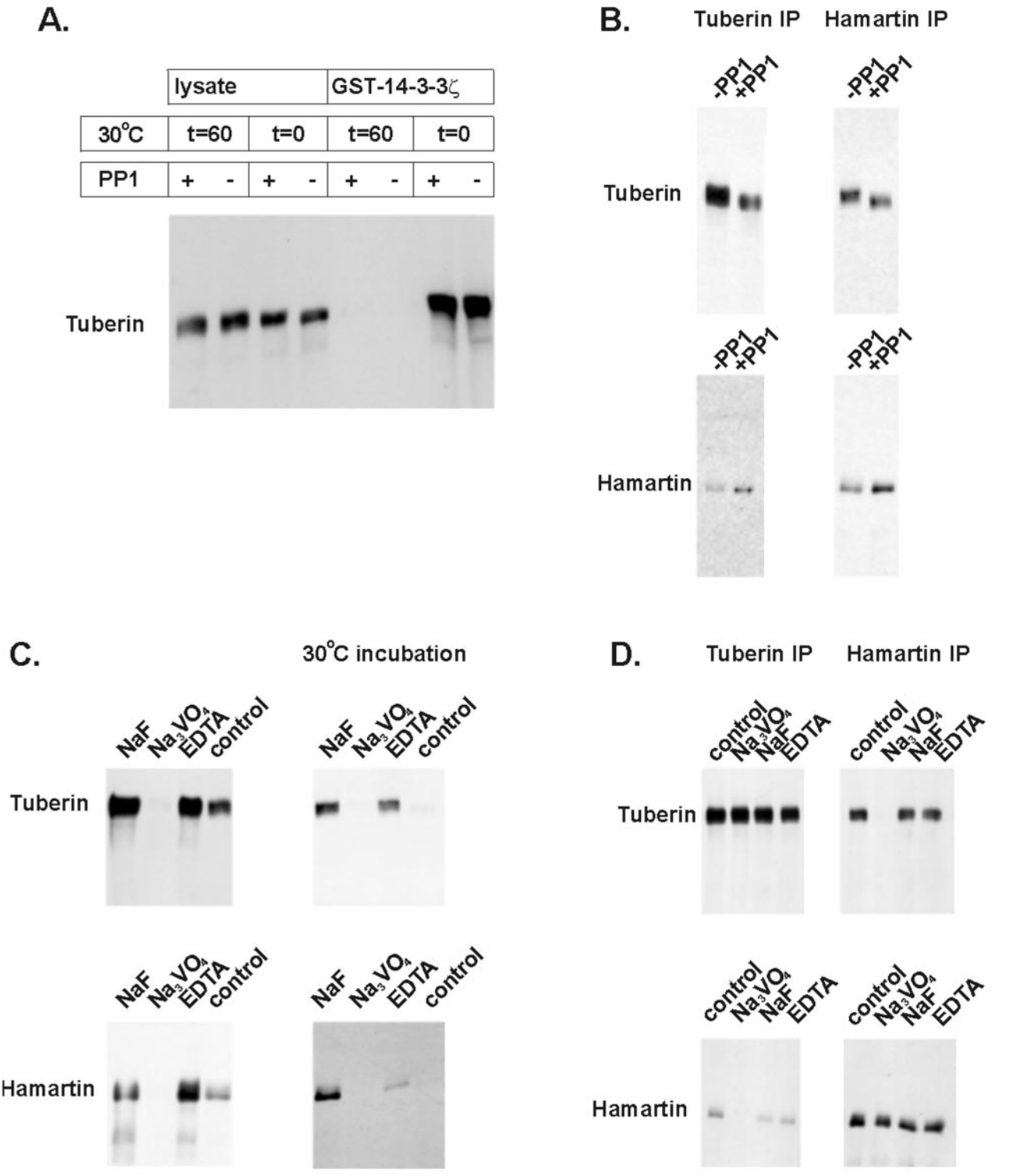

FIG. 6. **Tuberin phosphorylation is necessary for binding to 14-3-3ζ.** *A*, phosphatase activity prevents tuberin/14-3-3ζ binding. A cleared HeLa cell lysate was incubated at 30 °C for 60 min ($t = 60$) either with (+) or without (−) protein phosphatase-1 (*PP1*) before incubation with the GST-14-3-3ζ 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), Na_3VO_4 (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, Na_3VO_4, 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 Na_3VO_4 completely prevented co-immunoprecipitation of tuberin, indicating that Na_3VO_4 disrupted the tuberin-hamartin complex. Immunoprecipitation of either tuberin or hamartin alone was not affected by the presence of 10 mM Na_3VO_4.

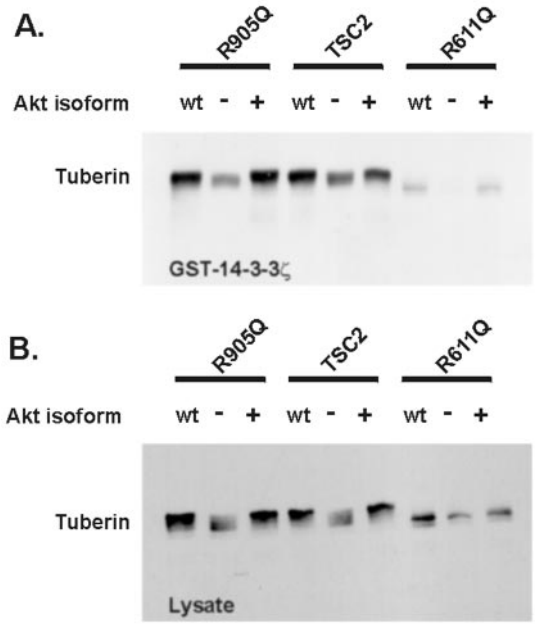

FIG. 7. **Akt-mediated phosphorylation regulates tuberin/14-3-3ζ binding.** COS-1 cell lysates overexpressing wild-type Akt (*wt*), a dominant-negative isoform (−), or a constitutively active isoform (+) together with wild-type tuberin (*TSC2*) or the R611Q or R905Q variant were incubated with the GST-14-3-3ζ 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.

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 as described above. As shown in Fig. 7, all three tuberin variants were expressed at similar levels; however, coexpression of the dominant-negative Akt isoform increased the mobility of wild-type tuberin and the R905Q variant on SDS-polyacrylamide gels, whereas the mobility of the R611Q variant was unaffected. This is consistent with previous studies suggesting that the tuberin R611Q variant is incorrectly phosphorylated (5).

As expected, the binding of wild-type tuberin and the R905Q variant to the GST-14-3-3ζ beads was more efficient than the binding of the R611Q variant. However, inhibition of Akt activity by expression of the dominant-negative Akt isoform reduced the binding of all three tuberin variants (the R611Q variant in particular) to the GST-14-3-3ζ beads relative to the binding when either wild-type Akt or the constitutively active isoform was expressed. Therefore, Akt activity promoted tuberin/14-3-3ζ binding, indicating that the interaction is dependent upon Akt-mediated phosphorylation of tuberin.

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 (R*X*R*XX*(S/T)) is similar to the RS*X*pS*X*P and R*XXX*pS*X*P consensus 14-3-3-binding motifs. Indeed, Scansite analysis predicts three putative Akt substrate motifs in tuberin (Ser^{939}, Ser^{981}, and Ser^{1338}) that are also potential 14-3-3-binding motifs. At least one of these sites (Ser^{939}) has been shown to be a primary site for Akt-dependent phosphorylation of tuberin (14). Therefore, following phosphorylation of tuberin by Akt, at Ser^{939} 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 only 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 phosphatase 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 Na_3VO_4, without affecting the direct immunoprecipitation of either protein, indicating that Na_3VO_4 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.

Acknowledgments—We thank Dr. N. Bottini for helpful discussions and Dr. J. Zhai, Professor J. L. Bos, Dr. M. van Triest, Dr. F. Tamanini, and Dr. E. van Herpen for providing reagents used in this study.

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

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J. Biol. Chem. 2002, 277:39417-39424.
doi: 10.1074/jbc.M204802200 originally published online August 9, 2002
