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Advances in Brief

14-3-3 Interacts with the Tumor Suppressor Tuberin at Akt Phosphorylation Site(s)[1]

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Abstract

Tuberin, the product of the tuberous sclerosis complex 2 tumor suppressor gene, is a phosphoprotein that negatively regulates phosphatidylinositol 3′-kinase signaling downstream of Akt. Several high stringency 14-3-3 binding sites that overlapped with Akt phosphorylation sites were identified in tuberin *in silico*. Recognition of tuberin by an α-14-3-3 binding site-specific antibody correlated with mitogen-induced phosphorylation of tuberin and recognition of tuberin by an α-Akt phosphorylation substrate antibody. Recognition of tuberin by both antibodies was blocked by inhibiting phosphatidylinositol 3′-kinase activity. Using a protein domain microarray, a tuberin peptide containing Ser^{939} demonstrated phospho-specific binding to 14-3-3. Glutathione *S*-transferase pull-down assays with 14-3-3 fusion proteins revealed that all seven 14-3-3 isoforms (β, γ, ζ, ε, τ, η, and σ) could bind tuberin, and this interaction was abrogated by competition with phosphorylated but not unphosphorylated Ser^{939} tuberin peptide. Tuberin also coimmunoprecipitated with 14-3-3, confirming the interaction between endogenous 14-3-3 and tuberin. These data establish the presence of functional and overlapping 14-3-3 and Akt recognition site(s) in tuberin.

Introduction

Signaling via cell surface receptors initiates the mitogenic response of cells to many growth factors and is a frequent target for events involved in the process of tumorigenesis. RTK[4] activation via growth factor stimulation or oncogenic mutation can initiate several phosphorylation cascades, often involving the production of second messengers such as PIPs (1). The PI3K signaling pathway is initiated by phosphorylation of PI3K via RTKs such as EGF receptor and platelet-derived growth factor receptor, activating PI3K, and resulting in production of PIP3 [PtdIns3P, PtdIns (3, 4, 5) P_3, and PtdIns (3, 4) P_2] (2). Formation of PIP3 recruits Akt/protein kinase B to the cell membrane where it is phosphorylated by PDK (3). Activation of Akt promotes cell survival and growth via downstream targets such as mTOR, nuclear factor κB, and glycogen synthase kinase-3β (2, 4–6). Signaling via mTOR results in activation of p70/S6K, which phosphorylates ribosomal protein S6 to promote protein synthesis and cell growth (7–10). The tumor suppressor gene *PTEN* exerts its activity by negatively regulating this pathway, functioning as a lipid phosphatase to dephosphorylate PIP3, and abrogating PI3K signaling (11–14). Loss of PTEN function in tumors and cell lines results in increased cell size and growth via constitutive activation of the PI3K/Akt pathway (15–17).

The *TSC-1* and *TSC-2* tumor suppressor genes have also recently been shown to modulate PI3K signaling downstream of Akt. TSC is an autosomal-dominant hereditary disease caused by loss of function of the *TSC-1* or *TSC-2* tumor suppressor genes (18, 19). TSC is characterized by the presence of hamartomatous and cystic lesions of the brain, heart, lungs, and kidney, and TSC patients are at increased risk for the development of renal cell carcinoma (19–21). The *TSC-1* and *TSC-2* gene products, hamartin and tuberin, are ubiquitously expressed and have been shown to interact and colocalize within cells (22–25). Epistasis studies in *Drosophila* were the first to suggest that dTSC1 and dTSC2 might regulate signaling by RTK (26–28), and both hamartin and tuberin have been directly demonstrated to negatively regulate the activity of P13K downstream from Akt (29, 30). Loss of tuberin or hamartin function results in constitutive activation of p70/S6K and phosphorylation of S6, leading to increased cell growth. This activity is sensitive to the mTOR inhibitor rapamycin, and reintroduction of tuberin into TSC-2-null cells suppresses p70/S6K activity, indicating that this tumor suppressor gene functions as an inhibitor of Akt and/or mTOR activity (29). Tuberin has been shown to be a phosphoprotein (31, 32), and global changes in phosphorylation, as detected by gel mobility shifts, have been shown to occur in response to serum stimulation (31). Most recently, tuberin has been shown to be a target for phosphorylation by Akt (33–36). We hypothesized that tuberin could participate in a regulatory loop in which phosphorylation by Akt relieved tuberin-mediated repression of Akt signaling by facilitating the interaction of tuberin with other cellular proteins such as 14-3-3.

Materials and Methods

Cells and Culture Conditions. MCF-7 (human breast cancer), NIH3T3 (mouse fibroblast), and TRKE-2 (rat kidney epithelial cell; Ref. 37) lines were grown in IMEM (Biosource International, Camarilla, CA), DMEM (Life Technologies, Inc., Rockville, MD), and DF8 complete medium, respectively, supplemented with 10% fetal bovine serum (Hyclone, Logan, UT) in a humidified 5% CO_2-95% air incubator at 37°C. The cells were cultured to 80% confluence, serum starved for 24 h, then stimulated for 1 h with medium containing 10% fetal bovine serum. For inhibitor experiments, NIH3T3 cells were pretreated with 20 μM LY294002 for 30 min before serum stimulation. PV to enhance phosphorylation was prepared by freshly mixing 100 μM Na_3VO_4 and 100 μM H_2O_2 together and treating NIH3T3 cells for 30 min.

Antibodies. 14-3-3γ (sc-731), normal rabbit IgG (sc-2027), and tuberin (sc-893) were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-phospho-Akt (cat no. 06-801) was from Upstate Biotechnology (Lake Placid, NY). Phospho-(Ser/Thr)-Akt substrate (no. 9611S), phospho-(Ser)-14-3-3 binding motif antibody (no. 9601S), phospho-S6 ribosomal protein antibody (no. 2211S), and S6 ribosomal protein antibody (no. 2212) were purchased from Cell Signaling Technology (Beverly, MA).

GST Fusion Protein Purification. pGEX-GST-14-3-3 constructs GST-14-3-3β, GST-14-3-3ζ, GST-14-3-3η, and GST-14-3-3τ were obtained from Dr. Ari Elson (Weizmann Institute of Science) and GST-14-3-3γ, GST-14-3-

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[4] The abbreviations used are: RTK, receptor tyrosine kinase; PIP, phosphatidylinositol phosphate; P13K, phosphatidylinositol 3′-kinase; EGF, epidermal growth factor; PtdIns, phosphatidylinositol; PDK, protein kinase D; mTOR, mammalian target of rapamycin; TSC, tuberous sclerosis complex; PV, pervanadate; GST, glutathione *S*-transferase; PBST, phosphate-buffered saline with Tween 20; PH, pleckstrin homology; S6K, S 6 kinase; TSC, tuberous sclerosis complex; IP, immunoprecipitate.

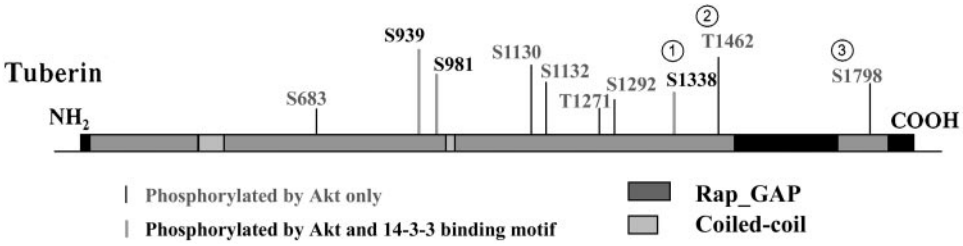

Fig. 1. Identification of putative Akt/14-3-3 sites in tuberin. Scansite generated line diagram of tuberin with domains and motifs indicated. The Rap GAP and coiled-coil domains are indicated. Akt phosphorylation and 14-3-3 binding sites identified are medium-high stringency. 1, human: 1338, rat: 1341, and mouse: 1340; 2, human: 1462, rat: 1466, and mouse: 1465; 3, human: 1798, rat: 1800, and mouse: 1805. The length of each bar is proportional to the stringency of the sites.

3ϵ, and GST-14-3-3σ constructs were obtained from Dr. Michael B. Yaffe (Massachusetts Institute of Technology). GST fusion proteins were overexpressed in *Escherichia coli* DH5α cells (Life Technologies, Inc.) by induction with a final concentration of 0.2 mM isopropyl-β-D-thiogalactopyranoside. Cells were broken by sonication. The resulting lysates were centrifuged, and the GST fusion proteins were then batch-purified from extracts by binding to glutathione Sepharose 4B beads (Amersham Pharmacia Biotech, Piscataway, NJ) and washed in PBS according to the manufacturer's instructions. The purified proteins were eluted from the beads with 30 mM glutathione, 50 mM Tris-HCl (pH 7.5), and 120 mM NaCl.

Production of Protein Microarrays. Protein domain microarrays were prepared as described previously (38). Briefly, proteins were arrayed in duplicate using a Flexys robot (Genomic Solutions, Ann Arbor, MI). The GST fusion proteins were arrayed from a 384-well plate, which contains 10 μl of each protein at a concentration of 1 μg/μl. Each protein was arrayed five times onto the same spot to increase the local concentration of protein. Proteins were spotted onto a glass slide precoated with nitrocellulose polymer (FAST Slide; Schleicher & Schuell, Keene, NH). The arrayed proteins were air-dried. Each block on the array contains 12 fusion proteins arrayed in duplicate, with GST alone spotted in the middle of the grid. Individual protein spots contain ~250 ng of fusion protein.

Labeling Peptide Probes with Cy5. Peptides were synthesized by the W. M. Keck Biotechnology Resource Center (New Haven, CT). Biotinylated peptides (10 μg) were prebound to 5 μl of Cy5-Streptavidin (Fluorolink; Amersham Pharmacia Biotech) in 500 μl of PBST. The fluorescent-labeled peptide was then incubated with 20 μl of biotin agarose beads (Sigma, St. Louis, MO) to remove the free Streptavidin label. Arrayed slides were blocked in PBST and 3% powdered milk, followed by the addition of 400 μl of fluorophore-tagged peptide. After 1 h of incubation at room temperature, the unbound peptide was washed away with PBST three times for 10 min each. After the washes, the slides were centrifuged dry, and the fluorescent signal was detected using a GeneTAC LSIV scanner (Genomic Solutions, Ann Arbor, MI). For controls, a 550 nm Long Pass Filter was used for the detection of FITC-conjugated secondary antibodies. A 675-nm Band Pass Filter was used for the detection of Cy5-labeled probes. Bound GST fusion protein was then detected by probing for 1 h with an αGST (Amersham Pharmacia Biotech), working at a dilution used for Western blot analysis (1:1000). The array was washed three times for 10 min in PBST. The primary antibody was recognized with an appropriate FITC-conjugated secondary antibody.

GST Pull-Down Assays. Serum-stimulated cells were washed, removed by scraping, resuspended in lysis buffer [20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM Na PP_I, 1 mM β-glycerolphosphate, 1 mM Na_3VO_4, 1 μg/ml leupetin, and 1 mM phenylmethylsulfonyl fluoride] and sonicated on ice. Protein (200 μg) from the cleared lysate was diluted in 350 μl of PBS with protease inhibitor mixture (¼ tablet of Complete; Roche in 50 ml of PBS), was mixed with the purified GST fusion protein (5–10 μg) bound to agarose beads and incubated 3 h at 4°C. Beads were washed in PBS and eluted in 30 μl of 1× SDS sample buffer (63 mM Tris-HCl, 10% glycerol, 2% SDS, and 0.0025% bromphenol blue) and heated at 99°C to release the bound protein, which was analyzed by Western blotting. For peptide competition assays, 200 μg of the above lysate was incubated with beads containing equal amounts of GST fusion protein or GST alone rocking for 3 h at 4°C in the presence or absence of 100 μM phospho-Ser^{939} or Ser^{939} tuberin peptide [SGSGFRARST***S*** (939) LNERPK].

Results

Screening of a Protein Domain Microarray Identifies a 14-3-3 Binding Site within Tuberin. To screen for potential sites of protein-protein interaction in tuberin, a Scansite[5] search was performed, which identified several overlapping 14-3-3 and Akt recognition sites. This program uses a motif profile scoring algorithm to compare all of the serine/threonine residues in the genome that lie within a RXRXXS/T (Akt) or a RSXpSXP consensus 14-3-3 binding site that overlaps with Akt phosphorylation motifs (39). In the case of Akt, each site is aligned with this optimal Akt phosphorylation motif and ranked according to how well it fits with this theoretical motif. As shown in Fig. 1, 10 putative Akt phosphorylation sites were identified on tuberin, 3 of which contained serines (Ser^{939}, Ser^{981}, and Ser^{1341}) that also overlapped with 14-3-3 sites. All three sites were highly conserved between human, rat, and mouse, consistent with a possible role in regulating tuberin function.

Using a novel protein domain microarray that we recently developed (38), candidate protein domains and motifs, including 14-3-3 proteins, were screened for their ability to interact with tuberin-specific peptides. Short linear tuberin sequence motifs predicted to bind 14-3-3 domains in a phospho-dependent manner were synthesized, labeled with Cy5, and used to probe a PH and 14-3-3 domain containing protein microarray. PH domains (40) were assessed as intensity controls (Fig. 2*A*, D and E first two blocks). All seven 14-3-3 isoforms were arrayed in the third block (Fig. 2*A*, D and E). An unphosphorylated peptide harboring residue Ser^{939} showed no specific binding to the 14-3-3 region of the array (Fig. 2*C*). However, when phosphorylated, this peptide did bind 14-3-3γ and, to a lesser extent, 14-3-3ζ (Fig. 2*B*). One of the PH domains was bound nonspecifically by both peptides in a phospho-independent fashion, serving as an internal control for the scan intensity. These results obtained on a nitrocellulose coated glass slide prompted us to further investigate the nature of the tuberin-14-3-3 interaction.

Tuberin Recognition by a 14-3-3 Substrate-specific Antibody Correlates with Phosphorylation by Akt. Tuberin displays decreased mobility in agarose gels when it is phosphorylated in response to conditions such as serum stimulation that stimulate PI3K (31). To determine whether this mobility shift correlates with PI3K/Akt signaling, NIH3T3 cells were serum starved and stimulated by serum or the phosphotase inhibitor PV in the presence or absence of the PI3K inhibitor LY294002. In response to 10% serum or PV (Fig. 3), tuberin showed a shift to the slower migrating hyperphosphorylated form. A total of 20 μM LY294002 pretreatment abrogated this phosphorylation, with tuberin remaining hypophosphorylated. Decreased tuberin mobility correlated well with activation of the PI3K/Akt pathway as monitored by increased phospho-Akt and phospho-S6 in the serum-stimulated and PV-treated cell lysates (Fig. 3), which was dramatically reduced in these cells by LY294002. Similar data were obtained for MDCK cells and NIH 3T3 cells stimulated by insulin-like growth

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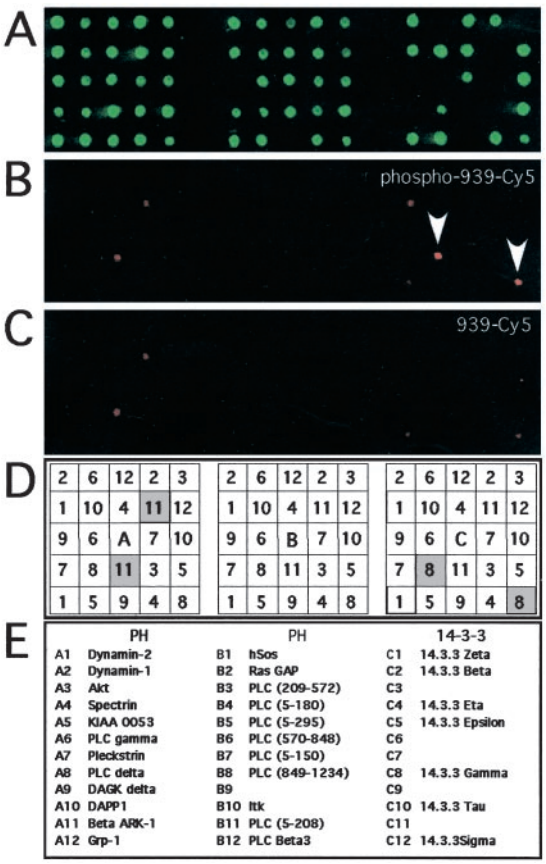

Fig. 2. Protein-domain microarray. Different GST fusion proteins were arrayed in duplicate onto a nitrocellulose slide. The arrays were probed with: *A*, αGST primary antibody and detected with a FITC-conjugated secondary antibody; *B*, Cy5-labeled tuberin phosphopeptide (biotin-SGSGFRARSTp**S**LNERPK); or *C*, Cy5-labeled unphosphorylated tuberin peptide (biotin-SGSGFRARSTSLNERPK). *D*, key to the arrayed domains. *Highlighted blocks* mark spots that are specifically bound in duplicate. The identity of the interacting domains can be extrapolated from *E*.

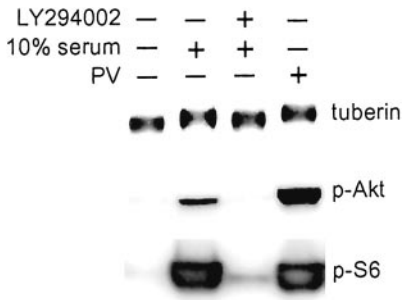

Fig. 3. Tuberin mobility shift correlates with Akt activation. *Top panel:* tuberin mobility shifts with treatment from fast, hypophosphorylated to slower, hyperphosphorylated form with a molecular weight of M_r 180,000–200,000. *Second and third panel:* levels of phospho-Akt and phospho-S6 in total cell lysates after treatment. Treatments are LY294002: PI3K inhibitor, PV: pervanadate.

factor I or EGF, which was abrogated by the EGF receptor inhibitor PD153035 (data not shown).

To confirm that tuberin phosphorylation correlated with activation of Akt, Western blotting was performed with a phospho-(Ser/Thr)-Akt substrate antibody. This antibody specifically recognizes peptides and proteins containing phospho-Ser/Thr preceded by Lys/Arg at position −5 and −3 (L/RXL/RXXpS/T), the suggested consensus site for Akt kinase phosphorylation (39, 41, 42). Tuberin was immunoprecipitated and probed with the *p*-Akt substrate antibody. As shown in Fig. 4 (*top panel*), tuberin from serum-starved NIH3T3 cells exhibited no detectable binding to the *p*-Akt substrate antibody, in contrast to serum or PV treatments, which dramatically increased phosphorylation of tuberin and recognition by the *p*-Akt substrate antibody. LY294002 treatment decreased Akt substrate antibody recognition of tuberin in serum-stimulated cells to basal (serum-starved) levels. To confirm that the IP products contained equal amounts of tuberin, membranes were stripped and reprobed using anti-tuberin antibody. (Fig. 4, *bottom panel*).

Membranes used to identify phospho-Akt substrate antibody binding were reprobed using a 14-3-3 mode 1 binding motif detection antibody. This antibody binds peptides and proteins containing a motif composed of phospho-serine contained within a 14-3-3 binding site with arginine or lysine at the −3 position. The recognition pattern was the same as the *p*-Akt substrate antibody: tuberin from serum-starved cells was barely recognized by the 14-3-3 binding motif antibody, whereas tuberin from lysates in which this protein was hyperphosphorylated were strongly recognized by this antibody (Fig. 4, *middle panel*). These data indicated that tuberin recognition by the 14-3-3 mode 1 binding motif detection antibody correlated with Akt activation and phosphorylation of tuberin.

Tuberin Binds 14-3-3 Isoforms *in Vitro* and *in Vivo*. To determine whether endogenous tuberin bound 14-3-3 protein(s), we performed a GST pull-down experiment with a complete set of GST-14-3-3 isoforms. Serum-stimulated NIH3T3 cell lysates were incubated with DH5α-expressed GST-14-3-3 fusion proteins prebound to glutathione-Sepharose 4B beads. Tuberin pulled down by the fusion proteins was detected by Western blotting. As shown in Fig. 5*A*, binding was seen to all of the seven isoforms of GST-14-3-3 but not to glutathione-Sepharose beads. Tuberin interaction with GST-14-3-3γ was additionally confirmed in cell lysates from tuberin-positive MCF-7 and TRKE-2 cell lines from human breast cancer and transformed rat kidney epithelial cells, respectively (Fig. 5*B*).

Because Ser^{939} of tuberin was predicted by Scansite to be a high-stringency site for phosphorylation by Akt and interaction with 14-3-3, phosphorylated and unphosphorylated Ser^{939} peptides were used to compete the interaction between tuberin and GST-14-3-3 fusion protein (Fig. 5*C*). Using lysates from serum-stimulated TRKE-2 cells, sephrose-bound GST-14-3-3γ was incubated with cell lysates in the presence or absence of $pSer^{939}$ or Ser^{939} and the immunoblots probed with α-tuberin antibody. In the presence of the *p*-Ser^{939} peptide, the tuberin signal was significantly reduced, whereas the unphosphorylated Ser^{939} peptide was unable to block the interaction between tuberin and 14-3-3γ, suggesting that phosphorylated Ser^{939} in tuberin

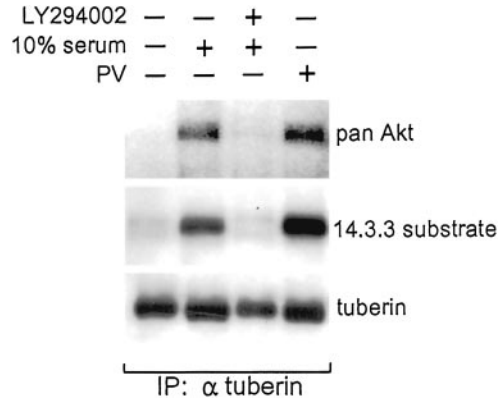

Fig. 4. Tuberin recognition by *p*-Akt substrate and 14-3-3 binding motif antibodies. Total cell lysates were prepared after indicated treatments and immunoprecipitated using antituberin antibody. IP product was probed using *p*-Akt-substrate antibody (*top panel*) or reprobed with 14-3-3 binding motif detection antibody (*middle panel*). After stripping, membranes were probed with α-tuberin as a control (*bottom panel*).

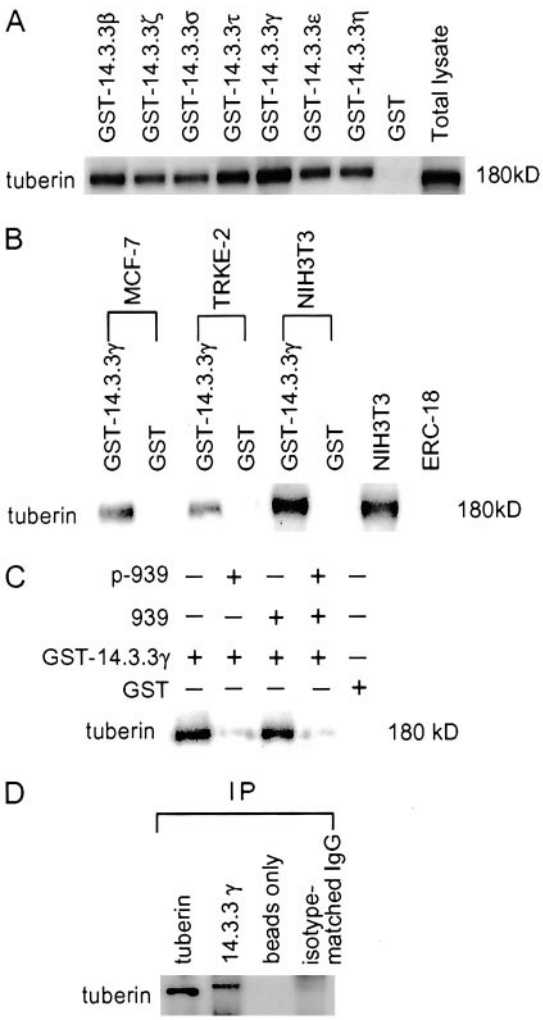

Fig. 5. 14-3-3 GST fusion proteins and endogenous 14-3-3 interact with tuberin. *A*, pulldowns of tuberin with 14-3-3 GST fusion protein probed for tuberin by Western blotting. Tuberin was significantly bound by all seven isoforms of GST-14-3-3. *B*, binding to tuberin by GST-14-3-3γ fusion protein in MCF-7, TRKE-2, and NIH3T3 lysates but not by GST beads alone. *C*, tuberin peptide competition for binding of 14-3-3 to tuberin from serum-stimulated TRKE-2 cells. Tuberin in TRKE cell lysates was reacted with GST-14-3-3γ beads in the presence or absence of 100 μM pSer^{939} or Ser^{939} tuberin peptide. Tuberin specifically associated with 14-3-3γ was detected by Western blotting. pSer^{939}, but not Ser^{939}, corresponding to phosphorylated serine at residue 939 in tuberin significantly blocks the interaction between tuberin and 14-3-3γ. *D*, coimmunoprecipitation demonstrating the interaction of endogenous 14-3-3 with tuberin. Lysates from TRKE2 cells were immunoprecipitated with 14-3-3γ antibody, and the IP product was Western blotted for tuberin detection. IP with only agarose beads (*third lane*) and with normal rabbit IgG and beads (*fourth lane*) served as negative controls.

is a major interaction site with 14-3-3γ. Tuberin could also be coimmunoprecipitated with anti-14-3-3γ, confirming the interaction of endogenous 14-3-3γ with tuberin (Fig. 5*D*). Tuberin that coprecipitated with 14-3-3 exhibited a slower mobility than tuberin present in total lysates, which correlated with 14-3-3γ binding preferentially with phosphorylated tuberin.

Discussion

Tuberin exists as a phosphoprotein, the target of both serine/threonine and tyrosine kinases (31). Our data are consistent with that of others, which indicate that at least one of these kinases is Akt, a downstream effector of the PI3K signaling pathway (33–36). Tuberin contains three recognition sites for Akt that are also potential 14-3-3 binding sites. Using a novel protein domain array, a tuberin peptide containing Ser^{939} bound to 14-3-3 in a phosphorylation-specific manner. All seven 14-3-3 isoforms recognized tuberin in GST-pull-down assays, and at least one of these, 14-3-3γ, recognized tuberin in fibroblasts (NIH3T3) as well as mammary (MCF-7) and kidney epithelial cells (TRKE) from mouse, human, and rat, respectively. The interaction between 14-3-3γ and tuberin was effectively competed with phosphorylated but not unphosphorylated Ser^{939} peptide. Endogenous tuberin could also be coimmunoprecipitated with 14-3-3 in kidney epithelial cell lysates. While in submission, our data indicating that tuberin interacts with 14-3-3 was confirmed by others (13–44).

14-3-3 molecules act as control points for many cellular processes (45–47) and often serve opposing functions, acting as scaffolding proteins to enhance the activity of proteins such as p53 (48, 49) or inactivating proteins such as BAD and Cdc25 by sequestration in the cytosol, partitioning these proteins into the cytoplasm to modulate nuclear import/export (45, 46). 14-3-3 ligand binding is often controlled by phosphorylation of serine or threonine residues contained within the optimum RSX(pS/pT)XP or RXXX(pS/pT)XP 14-3-3 recognition sites (50–52). The *RSXpS*XP 14-3-3 recognition sequence can overlap with the Akt consensus sequence, RX*RXX(pS/pT)*, and thus in certain cases, proteins phosphorylated by Akt are recognized by 14-3-3 in response to Akt phosphorylation. Proteins targeted by Akt and recognized by 14-3-3 include FKHRL (53–57), BAD (58, 59), Raf (60–64), and IRS-1 (65–68). Ser^{939} of tuberin is contained within the sequence RSXpSX, which fits the Akt/14-3-3 consensus and is conserved between human, rat, and mouse. Although this recognition site lacks a P two positions COOH-terminal to Ser^{939}, this residue is not absolutely required for peptide or protein binding to 14-3-3 (69). For most proteins with multiple 14-3-3 sites, one site appears to be dominant, with secondary sites unable to promote a stable 14-3-3 interaction when the dominant site is absent or not phosphorylated (46). While it is clear that tuberin can be phosphorylated at multiple sites, peptide competition experiments performed with the Ser^{939} phosphopeptide would suggest that this may be a dominant 14-3-3 binding site, as the Ser^{939} peptide effectively competed for the vast majority of tuberin recognized by 14-3-3.

The function of tuberin bound to 14-3-3 is not known at this time. 14-3-3 proteins can both activate and inactivate their ligands, depending on the target protein (46). Tuberin has recently been shown by us and others to be a negative regulator of Akt signaling via mTOR and/or p70/S6K (29, 33–36). Loss of Tsc-2 gene function relieves tuberin-mediated suppression of Akt activity, resulting in increased activation of S6 (29, 33–36), a major control point for protein synthesis and cell growth. In support of this model, tuberin has recently been shown to be constitutively phosphorylated in PTEN$^{-/-}$ tumor-derived cell lines in which Akt becomes constitutively active (33). It is attractive to speculate that phosphorylation of tuberin by Akt participates in a negative regulatory loop in which phosphorylation of tuberin results in sequestration by 14-3-3, abrogating the inhibitory effect of this tumor suppressor gene on Akt signaling.

Acknowledgments

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