Tuberin Regulates p70 S6 Kinase Activation and Ribosomal Protein S6 Phosphorylation

A ROLE FOR THE TSC2 TUMOR SUPPRESSOR GENE IN PULMONARY LYMPHANGIOLEIOMYOMATOSIS (LAM)*

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Although the cellular functions of TSC2 and its protein product, tuberin, are not known, somatic mutations in the TSC2 tumor suppressor gene are associated with tumor development in lymphangioleiomyomatosis (LAM). We found that ribosomal protein S6 (S6), which exerts translational control of protein synthesis and is required for cell growth, is hyperphosphorylated in the smooth muscle-like cell lesions of LAM patients compared with smooth muscle cells from normal human blood vessels and trachea. Smooth muscle (SM) cells derived from these lesions (LAMD-SM) also exhibited S6 hyperphosphorylation, constitutive activation of p70 S6 kinase (p70S6K), and increased basal DNA synthesis. In parallel, TSC2−/− smooth muscle cells (ELT3) and TSC2−/− epithelial cells (ERC15) also exhibited hyperphosphorylation of S6, constitutive activation of p70S6K, and increased basal DNA synthesis. Re-introduction of wild type tuberin into LAMD-SM, ELT3, and ERC15 cells abolished phosphorylation of S6 and significantly inhibited p70S6K activity and DNA synthesis. Rapamycin, an immunosuppressant, inhibited hyperphosphorylation of S6, p70S6K activation, and DNA synthesis in LAMD-SM cells. Interestingly, the basal levels of phosphatidylinositol 3-kinase, Akt/protein kinase B, and p42/p44 MAPK activation were unchanged in LAMD-SM cells. These data demonstrate that tuberin negatively regulates the activity of S6 and p70S6K specifically, and suggest a potential mechanism for abnormal cell growth in LAM.

Lymphangioleiomyomatosis (LAM)† is a disorder character-
ized by benign lesions of smooth muscle-like cells in the lung (1–5). These lesions promote cystic destruction of the lung and lead to loss of pulmonary function, for which there is no therapy (6). The molecular mechanisms promoting LAM cell proliferation remain largely unknown (7). Genetic studies demonstrate that somatic mutations in the tumor suppressor TSC2 gene are associated with pulmonary LAM (8–10).

Evidence suggests that TSC2 gene activation alters the proliferation of mammalian cells. Soucek et al. (11), using antisense oligonucleotides, showed that tuberin negatively regulates the G1/S transition of immortalized rat fibroblast cell line Rat1. Mutations in the Drosophila homolog of TSC2 gene increased cell growth and shortened the G1 phase of the cell cycle, which was comparable with results obtained by overexpressing phosphatidylinositol 3-kinase (PI 3-kinase), Myc, or Ras (12–14). Mice lacking tuberin die as embryos and show hypoplasia of the liver and developmental abnormalities of other abdominal organs (15, 16). Heterozygous mice develop cysts and slow growing tumors in a variety of tissues. The Eker rat, a model for hereditary renal cancer, has an insertion in the TSC2 locus resulting in a tuberin mutation and tumor development in multiple organs (17, 18). Thus, as in the human disease, loss or mutation of TSC2 modulates cell proliferation.

TSC2 encodes a 1784-amino acid 200-kDa protein, tuberin, that contains a region with sequence similarity to the GTPase-activating protein (GAP) for Rap1 GTPase (19). The putative GAP domain has been reported to increase the intrinsic GTPase activity of both Rab5 and Rap1A GTPases (20, 21). Further downstream, tuberin modulates the transcriptional activity of AP1 and the steroid hormone receptor family (22–24). However, little else is known about how tuberin is regulated or about its other downstream signaling targets.

Given the phenotypic similarity between loss of TSC2 and overexpression of PI 3-kinase in Drosophila (25), we hypothesized that deregulation of PI 3-kinase effectors might be im-

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‡ The abbreviations used are: LAM, lymphangioleiomyomatosis; PI 3-kinase, phosphatidylinositol 3-kinase; GAP, GTPase-activating protein; MAPK, mitogen-activated protein kinase; p70S6K, p70 S6 kinase; mTOR, mammalian target of rapamycin; SM, smooth muscle; FBS, fetal bovine serum; ASM cells, airway smooth muscle cells; VSM cells, vascular smooth muscle cells; HLF, human lung fibroblasts; GFP, green fluorescent protein; TBS, Tris-buffered saline; BrdUrd, 5-bromo-2′-deoxyuridine, MOPS, 4-morpholinepropanesulfonic acid; ANOVA, analysis of variance.

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portant for the abnormal cell proliferation associated with TSC2 deficiency. One critical downstream target of PI 3-kinase, p70 S6 kinase (p70S6K), affects cell and organ size in Drosophila (26). Activation of p70S6K and its subsequent phosphorylation of ribosomal protein S6 are required for biosynthesis of the cellular translational apparatus, a critical component for cell growth and proliferation. Conditional deletion of the S6 gene in the liver of adult mice abrogates cell proliferation and cyclin E expression despite the formation of cyclin D-CDK4 complexes (27). The central role of p70S6K and ribosomal protein S6 in cellular proliferation has been further demonstrated by the use of rapamycin, a macrolide that specifically and directly inhibits the mammalian target of rapamycin (mTOR), an obligated upstream activator of p70S6K (28–30).

In the current study, we demonstrate that tuberin specifically regulates the activity of p70S6K and ribosomal protein S6. S6 hyperphosphorylation and p70S6K activation and increased DNA synthesis were observed in LAM tissue samples, primary LAM-derived smooth muscle (LAMD-SM) cultures, and established cell lines lacking the functional tuberin protein. These effects were reversed by the re-introduction of tuberin. Surprisingly, other signaling molecules such as PI 3-kinase, Akt, and MAPK were unaffected by cellular levels of tuberin. These results suggest that tuberin may modulate its effects on cellular proliferation through specific modulation of the p70S6K pathway.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and TSC2 Mutational Analysis of LAMD-SM Cells—** LAMD-SM cells were dissociated from LAM nodules obtained from the lungs of LAM patients who have undergone lung transplant. LAM tissue was obtained in compliance with the University of Pennsylvania Institutional Review Board approved protocol and the protocol approved by NHLBI, National Institutes of Health LAM Registry Tissue Committee. Briefly, nodules were subjected to an enzymatic digestion in 10 ml of M199 medium containing 0.2 mM CaCl₂, 2 mg/ml collagenase D (Roche Molecular Biochemicals), 1 mg/ml trypsin inhibitor (Sigma), and 3 mg/ml elastase (Worthington, Lakewood, NJ) for 60 min in a shaking water bath at 37 °C. The cell suspension was filtered and then washed to equal volumes of cold DF8 medium containing of equal amounts of Ham’s F-12 and Dulbecco’s modified Eagle’s medium with 1.6 × 10⁻³ M ferrous sulfate, 1.2 × 10⁻⁵ units/ml vasopressin, 1.0 × 10⁻⁵ M triiodothyronine, 0.025 mg/ml insulin, 1.0 × 10⁻⁶ M cholesterol, 2.0 × 10⁻⁵ M hydrocortisone, and 10 pg/ml transferrin supplemented with 10% FBS. Aliquots of the cell suspension were plated at a density of 1.0 × 10⁴ on tissue culture plates coated with Vitrogen (Cohesion Technologies Inc., Palo Alto, CA). The cells were cultured in DF8 medium and were passaged twice a week. LAMD-SM cells in subculture during the third through twelfth cell passages were used. All experiments were performed using in parallel three primary cell lines derived from two LAM patients. LAMD-SM-1 cell line represents the total population of cells derived from one nodule of one patient. LAMD-SM-2 and LAMD-SM-7 are clonal lines, two of eight clones that were derived from a LAM nodule of another patient. Each cell line was characterized on the basis of TSC2 mutational analysis, tuberin expression, smooth muscle α-actin expression, the level of DNA synthesis, and HMBS immunoactivity. TSC2 gene mutational analysis of these cell lines was performed on DNA and RNA samples by two methods. First, reverse transcriptase-PCR analysis of the entire coding region of the TSC2 mRNA was performed using a set of eight primer pairs to generate eight overlapping fragments (31). These fragments were subjected to bi-directional sequencing and compared with the TSC2 consensus sequence using the program Sequencher version 2.0. Analysis of selected individual exons of TSC2 by denaturing high-performance liquid chromatography and sequencing was also performed as described (31). Comparative analysis of morphological, biochemical, and mutational characteristics demonstrated similarities between LAMD-SM-1, LAMD-SM-2, and LAMD-SM-7 cell lines. Morphologically LAMD-SM cells were spindle-like with high levels of basal DNA synthesis (see “Results”), and they showed positive immunoactivity to anti-smooth muscle α-actin antibody (data not shown) and tuberin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) (See “Results”). However, LAMD-SM cells showed no immunoactivity for HMBS-45 (DAKO Co., Carpinteria, CA) (data not shown), which showed positive immunostaining of the epithelial cells in LAM nodules (data not shown) (3, 4, 32) and in human melanoma, used as a positive control. All assays were performed on cells maintained for 48 h in serum-free DF basal media containing 1% bovine serum albumin before starting the experiment.

**Cell Culture of TSC2+/− and TSC2+/+ Cells—** ELT3 smooth muscle cell line was derived from the Eker rat uterine leiomyoma (33). The TSC2+/− ERC15 cell line was derived from an Eker rat renal carcinoma (34). The TSC2+/+ TRKE2 cell line was derived from rat primary kidney epithelial cells (35). ELT3, ERC15, and TRKE2 cells were maintained in DF8 medium (33). Human airway smooth muscle cells (HASM), primary human arterial vascular smooth muscle cells, and human lung fibroblasts (HLF) were used as TSC2+/− cell lines. Expression of tuberin in these cells was confirmed by immunoblot analysis with anti-tuberin antibody (data not shown). ASM and VSM cells and HLF were dissociated from human trachea, human pulmonary artery, and human lung, respectively, which were obtained from human lung transplant donors and were previously described (36, 37). ASM cells were maintained in Ham’s F-12 with 10% FBS; VSM were maintained in Ham’s F-12 with 10% FBS supplemented with 15 μg/ml endothelial cell growth supplement (BD Biosciences), HLF were maintained in RPMI supplemented with 10% FBS. All assays were performed on cells maintained for 48 h in serum-free medium before starting the experiment. ELT3 and ERC15 were maintained in serum-free Ham’s F-12 basal media containing 1% bovine serum albumin; ASM and VSM cells were in serum-free Ham’s F-12 supplemented with 0.1% bovine serum albumin.

**Microinjection and Immunofluorescent Staining—** Microinjection was performed using Eppendorf Microinjection System (Hamburg, Germany). Briefly, cells plated on 2-well glass chamber slides (Nalgene Nunc International, Naperville, IL) and maintained in serum-free medium were microinjected with pEGFP or pEGFP-TSC2 plasmids expressing green fluorescent protein (GFP) or GFP-tagged tuberin, respectively, or with inhibitory anti-VPS34 antibody, which specifically recognize and inhibit class III PI 3-kinase (38) simultaneously with dextran Rhodamine Green (Molecular Probes, Eugene, OR) to identify microinjected cells. Eighteen hours after injection of plasmids or 30 min after the injection of antibody, the cells were washed 3 times in phosphate-buffered saline, fixed with 3.7% paraformaldehyde (Polysciences, Inc, Warrington, PA) for 15 min, and treated with 0.1% Triton X-100 (Sigma) for 30 min at room temperature. The cells were blocked with 0.5% TSA Flouresce™ System blocking reagent (PerkinElmer Life Sciences) in 20 μM Tris (pH 7.5) and 150 mM NaCl (TBS) for 1 h at 37 °C. After incubation with primary antibodies (anti-phospho-ribosomal protein S6 (RPS6) antibody (Upstate Biotechnology, Lake Placid, NY, 1:50) or anti-GFP, rabbit serum (Molecular Probes; 1:200 dilution) and then secondary antibodies (Alexa Fluor 594 donkey anti-tuberin IgG conjugate, 1:400 dilution, or Alexa Fluor 488 goat anti-rabbit IgG conjugate (Molecular Probes) 1:400) for 1 h at 37 °C, the cells were mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA). The cells were visualized using a Bio-Rad 1024-MF confocal microscopic system with Nikon Eclipse E400 microscope under appropriate fluorescence microscope.

**Immunohistochemical Analysis—** LAM tissue sections were stained with hematoxylin and eosin or immunohistochemically with anti-smooth muscle α-actin clone 1A4 fluorescein isothiocyanate conjugate (Sigma), primary anti-phospho-ribosomal protein S6 antibody (Upstate Biotechnology), and secondary Alexa Fluor 594 donkey anti-sheep IgG conjugate (Molecular Probes) antibodies. Negative controls included omission of the primary antibody or replacement of the primary antibody with isotype matched IgG.

**Transient Transfection—** Plasmids were prepared using EndoFree Plasmid Maxi Kit (Qiagen Inc., Valencia, CA). Transient transfection was performed using the Effectene transfection reagent (Qiagen) according to the manufacturer’s protocol. Briefly, cells were incubated with pcDNA3 or pcDNA3-TSC2 (21, 39) for 6 h and then washed with phosphate-buffered saline and maintained for the next 24 h in medium supplemented with 10% FBS. Cells were maintained for 48 h in serum-free media before the immunoblot, p70S6K activity, or DNA synthesis assays. Transient transfection of pcDNA-TSC2 plasmid was verified by immunoblot assay using anti-tuberin (C20) (Santa Cruz Biotechnology) antibody.

**DNA Synthesis—** Near confluent cells, grown on 2-well glass chamber slides, were maintained for 48 h in serum-free medium, then after 16 h, 10 μM 5-bromo-2-deoxyuridine (BrdUrd), a thymidine analogue, was added to all wells. Twenty-four hours after the addition of BrdUrd, the cell monolayers were fixed with 3.7% paraformaldehyde and then permeabilized with 0.1% Triton X-100. After denaturation of DNA with 4 N HCl for 3 min at room temperature, the monolayers were incubated with 0.5% TSA Fluoresce™ System blocking reagent (PerkinElmar Life Sciences) in 20 μM Tris (pH 7.5) and 150 mM NaCl (TBS) for 1 h at 37 °C. After incubation with primary antibodies (anti-phospho-ribosomal protein S6 (RPS6) antibody (Upstate Biotechnology, Lake Placid, NY, 1:50) or anti-GFP, rabbit serum (Molecular Probes; 1:200 dilution) and then secondary antibodies (Alexa Fluor 594 donkey anti-sheep IgG conjugate, 1:400 dilution, or Alexa Fluor 488 goat anti-rabbit IgG conjugate (Molecular Probes) 1:400) for 1 h at 37 °C, the cells were mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA). The cells were visualized using a Bio-Rad 1024-MF confocal microscopic system with Nikon Eclipse E400 microscope under appropriate fluorescence microscope.
1 at 37 °C with 2 μg/ml mouse anti-BrdUrd antibody (BD Biosciences) and then with 10 μg/ml Texas Red conjugated anti-mouse antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 h at 37 °C to detect BrdUrd-positive cells. The cells were then incubated with 4,6-diamidino-2-phenylindole (1 μg/ml) in 0.5% NaCl to detect the total number of nuclei. The cells were examined using a fluorescent microscope (Nikon Eclipse E400) under 200-fold magnification with the appropriate fluorescent filters. Mitotic index was defined as the percentage of BrdUrd-positive nuclei/total number of cells/field. A total of 200 cells were counted/each condition in each experiment.

Preparation of Cell Lysates and Immunoblot Analysis—Cells were washed with PBS, lysed in NP-40 lysis buffer (25 mM Tris-base (pH 7.4), 150 mM NaCl, 0.5% sodium deoxycholate, 1.0% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 10 mM β-glycerophosphate, 1% Nonidet P-40, 1 mM benzamidine, 10 mM p-nitrophenylphosphate, 0.1 mM phenylmethylsulfonyl fluoride (Sigma)) or radioimmunoprecipitation buffer-TBS lysis buffer (25 mM Tris-base HCl (pH 7.4), 150 mM NaCl, 0.5% sodium deoxycholate, 1.0% Nonidet P-40 (Calbiochem), 0.1% SDS, 1 mM EGTA, 5 mM EDTA, 200 μM Na3VO4, 10 μg/ml aprotinin, 10 μg/ml leupeptin (Sigma), 1 mM phenylmethylsulfonyl fluoride) for 30 min at 4 °C. Frozen tissue samples were homogenized with radioimmunoprecipitation buffer-TBS lysis buffer and lysed for 30 min at 4 °C. The lysates were centrifuged at 14,000 rpm for 10 min at 4 °C. Protein contents were measured using a Bio-Rad protein assay reagent kit (Bio-Rad). Equal amounts of lysate, adjusted to protein content, were subjected to SDS-PAGE and immunoblot analysis. The blots were exposed to either anti-p70 S6 kinase, anti-tuberin (C20) sc-893 (Santa Cruz Biotechnology), anti-phospho-p70S6K (Thr-389) (Upstate Biotechnology), anti-phospho-p70S6 kinase (Thr-421/Thr-424), anti-S6 ribosomal protein, anti-phospho-Akt (Ser-473), anti-Akt (Cell Signaling Technology, Inc., Beverly, MA), anti-phospho-p42/p44 MAPK (Thr-202/Tyr-204), or anti-p42/p44 MAPK (New England Biolabs, Inc., Beverly, MA) antibodies. All antibodies were in TBS plus 0.5% Tween 20 (TBST), and all incubations were overnight at 4 °C. After three washes in TBST, the nitrocellulose filters were exposed to either anti-rabbit, anti-mouse (Boehringer-Mannheim) or anti-sheep (Upstate Biotechnology) horseradish peroxidase-conjugated secondary antibodies. Filters were washed five times in TBST and visualized using Enhanced Chemiluminescence (ECL) (Amersham Biosciences).

Image analysis was performed using the Gel-Pro analyzer program (Media Cybernetics, Silver Spring, MD).

*p70S6K Activity—In vitro p70S6K activity assay was performed as described previously (40). Briefly, serum-free medium-maintained cells were washed twice in ice-cold phosphate-buffered saline and then lysed in lysis buffer (20 mM Tris (pH 8.0), 150 mM NaCl, 5 mM EGTA, 1 mM EDTA, 1 μg/ml leupeptin, 1% Nonidet P-40, 10 mM p-nitrophenylphosphate, 0.1 mM phenylmethylsulfonyl fluoride). After incubation for 30 min at 4 °C, lysates were centrifuged at 14,000 rpm for 10 min. Supernatants were incubated with 2 μg of anti-p70S6K antibody with gentle rocking overnight at 4 °C. The immunocomplexes were collected by 50 μl of protein A-Sepharose, 50 μl of protein A-Sepharose beads were then resuspended in assay dilution buffer containing 50 μM substrate peptide, 4 μM protein kinase C inhibitor peptide, 0.4 μM protein kinase A inhibitor peptide, and γ32P-ATP (PerkinElmer Life Sciences). The samples were incubated for 10 min at 30 °C, and then 20 μl of the reaction mixture was spotted onto ps1 phosphocellulose filters, which were washed 3 times with 0.75% phosphoric acid and 1 time with acetone. The radioactivity of samples was measured using a Beckman LS 6500 scintillation counter.

Phosphatidylinositol 3-Kinase Activity—Phosphatidylinositol 3-kinase activity assays were performed as previously described (41). The cells were washed twice with ice-cold wash buffer (137 mM NaCl, 20 mM Tris-HCl, 1 mM MgCl2, 1 mM CaCl2, 0.2 mM Na3VO4, 10 mM β-glycerophosphate (Sigma)) and lyased in lysis buffer (wash buffer plus 10% (v/v) glycerol, 1% (w/v) Nonidet P-40, 1 mM phenethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin (42)). The lysates were centrifuged at 14,000 rpm for 10 min. Supernatants were incubated with anti-phosphotyrosine (Upstate Biotechnology), (2 μg/ml), anti-p85α (2 μg/ml) (Upstate Biotechnology), anti-p110α (2 μg/ml) (Santa Cruz Biotechnology), or anti-p110δ (2 μg/ml) (Santa Cruz Biotechnology) antibodies. Protein A-Sepharose, 50 μl was then added to the lysates for 2 h at 4 °C. The immunoprecipitates were washed 3 times in phosphate-buffered saline containing 1% Nonidet P-40, 2 times in 0.1% Triton-HCl (pH 7.5), 0.5% LiCl, and 2 times in TNE (10 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA (pH 7.5)). All solutions contained 0.2 mM vanadate. Sonicated phosphatidylinositol (Sigma) in Tris-HCl/EGTA (0.2 μg/ml, final concentration) was added to immunoprecipitates, and the phosphorylation reactions were started by the addition of MgCl2, ATP, and γ32P-ATP (30 μCi/sample) for 10 min at 30 °C. Reactions were stopped by the addition of 100 μl of 1 N HCl and extracted with 160 μl of chloroform-methanol (1:1). Lipids were separated on oxalate-coated TLC plates (Merck) using a solvent system of chloroform-methanol-water-ammonium hydroxide (60:40:11.3:2) and then detected by autoradiography. The position of [32P]Phosphatidylinositol 3-phosphonate was determined by the position of a phosphatidylinositol phosphate standard that is separated on a TLC in parallel and developed in iodine vapor.

Data Analysis—Data points from individual assays represent the mean ± S.E. Statistically significant differences among groups were assessed with the analysis of variance (ANOVA) (Bonferroni Dunn test), with values of p < 0.05 sufficient to reject the null hypothesis for all analyses. All experiments were designed with matched control conditions within each experiment to enable statistical comparison as paired samples.

Results

Mutational Analysis of TSC2 Gene in LAMD-SM Cells—Because somatic mutation of the tumor suppressor TSC2 gene is associated with LAM disease, mutational analysis of the gene in LAMD-SM cells derived from LAM patients was performed and compared with the location of the mutation in the Eker rat and the mutations in Drosophila affecting cell growth. The TSC2 gene, comprising 41 exons, encodes for tuberin, a 200-kDa protein with predicted leucine zipper motif at amino acids 81–98 (exon 3), two small coiled-coil domains at amino acids 346–371 (exon 10) and 1008–1021 (exon 26), and a small region of homology to the Rap1 GAP at amino acids 1593–1878 (exons 34–38) (19, 43, 44) (Fig. 1). Mutational analysis of the TSC2 gene in LAMD-SM cells was performed on DNA and RNA derived from these cell cultures. Analysis of LAMD-SM-1 cells by reverse transcriptase-PCR indicated the presence of the rare variant C2580T in exon 22 of TSC2. This polymorphism has been seen previously at a frequency of 0.5% in TSC2 (zk.bwh.harvard.edu/projects/tsc/polymorphisms.html), suggesting that it was present in the hemizygous state in LAMD-SM-1 cells and that there was loss of one TSC2 allele due to a large genomic deletion. Further evidence for this conclusion was the observation that the TSC2 sequence of LAMD-SM-1 was homo/hemizygous for the rare allele of several additional polymorphic variants in the 3′-half of TSC2. Analysis of LAMD-SM-2 and LAMD-SM-7 cells by reverse transcriptase-PCR demonstrated
that there were equal amounts of two different TSC2 mRNA isoforms (data not shown). One was the normal transcript, whereas the other was missing exon 10, which would result in the deletion of 48 amino acids, 322–370, from the tuberin sequence. Analysis of DNA from LAMD-SM-2 and LAMD-SM-7 demonstrated that exon 10 contained the mutation of cysteine 1108 to threonine (C1108T), which results in nonsense mutation (X) of glutamate at residue 370 (Q370X), and in converting its triplet nucleotide sequence to a stop codon. This nonsense mutation is similar to many other nonsense mutations in TSC2 that are thought to be inactivating (7) (expmed.bwh.harvard.edu/resolve). These data demonstrate that one allele of TSC2 gene is mutated in cells derived from nodules of LAM patients. Immunoblot analysis of whole cell lysates of LAMD-SM-1, LAMD-SM-2, and LAMD-SM-7 cells as well as of control primary cultures of TSC2++/+ HLF and ASM, and VSM cells demonstrated that tuberin was expressed in all cell lines (Fig. 2). Interestingly, the tuberin levels in LAMD-SM-2 and LAMD-SM-7 cells tend to be lower compared with levels in LAMD-SM-1 or ASM cells.

In the Eker rat a germ line insertion mutation (intron 30) of the TSC2 gene causes structural alteration of the TSC2 gene, which results in encoding truncated tuberin, which is one-third shorter than normal tuberin (17, 18, 45) (Fig. 1). The TSC2−/− ELT3 and ERC15 cell lines used in our study were derived from spontaneous tumors in the Eker rat. ELT3 cells show loss of heterozygosity at the TSC2 locus, implying a loss of the remaining wild type allele (33, 34). These cells contain one Eker mutant TSC2 gene; thus, the net effect is a “two-hit” inactivation and loss of tuberin expression (46, 47). ERC15 cells do not show loss of heterozygosity at the TSC2 locus, but no tuberin is detected by immunoblot analysis, which may be due to other mechanisms of second hit inactivation. The Drosophila homolog of TSC2 gigas, comprising 16 exons, encodes protein Gigas, which is 26% identical (46% similar) to the human protein tuberin, with the highest level of conservation (53% identity) spanning the GAP domain (12). Cells mutant for gigas are increased in size with unaffected differentiation (12). Collectively, our data and published evidence suggest that TSC2 gene mutations affect cell growth.

Ribosomal Protein S6 Is Hyperphosphorylated in LAM Nodules—To determine whether an in vivo correlation exists between the activation of ribosomal protein S6 and LAM disease, which is associated with somatic mutations of TSC2 gene (8–10), immunohistochemical analysis was used to identify atypical smooth muscle cells in LAM nodules and determine their relative levels of ribosomal protein S6 phosphorylation. The histopathological appearance of the LAM lung tissue, determined by hematoxylin and eosin staining, showed spindle cell nodules in the lungs compared with normal lung tissue (Fig. 3). The LAM cells formed nodules of varying size that were scattered in lungs. The smooth muscle cells in the LAM nodules markedly express smooth muscle α-actin (Fig. 3). In contrast, smooth muscle α-actin expression in normal lung was seen only in the smooth muscle cells of the blood vessel walls (Fig. 3).

In parallel experiments, activation of ribosomal protein S6 was determined by examining its phosphorylation state. Phosphorylation of S6 on multiple sites stimulates its ability to promote translation. In LAM nodules, many of the smooth muscle cells stained positively for ribosomal protein S6 phosphorylation (Fig. 4, upper panels). In contrast, smooth muscle cells from blood vessels (Fig. 4, lower panels) and normal human trachea (not shown) exhibited negligible phospho-ribosomal protein S6 immunoreactivity. These data demonstrate that ribosomal protein S6 is activated in smooth-muscle-positive cells in LAM nodules.

Ribosomal Protein S6 Is Hyperphosphorylated in LAMD-SM and TSC2−/− Cells—The immunohistochemical results above suggest that tuberin deficiency results in the hyperphosphorylation of ribosomal protein S6. To confirm this observation, S6 phosphorylation was examined in established cell lines, primary cultures, and tissue samples by immunoblot analysis. As seen in Fig. 5A, S6 was hyperphosphorylated in LAM-derived tissue relative to tissue samples taken from normal lung, bronchus, or pulmonary artery. Similarly, strong activation of S6 was observed in primary cultures of LAMD-SM (Fig. 5B). In contrast, the level of S6 phosphorylation was markedly lower in primary cultures of TSC2++/+ human lung HLF and ASM, and VSM cells (Fig. 5B). Finally, in rat ELT3 and ERC15 cells, two established TSC2−/− cell lines, S6 was strongly phosphorylated compared with rat TSC2++ TRKE2 cells (Fig. 5B). Furthermore, immunostaining of LAMD-SM, ELT3, and ERC15 cells also demonstrated activation of S6 (data not shown). Taken together, these data establish a correlation between tuberin deficiency and hyperphosphorylation of S6.

Expression of TSC2 Attenuates Hyperphosphorylation of Ribosomal Protein S6 in LAMD-SM and TSC2−/− Cells—We next examined whether expression of tuberin in LAMD-SM and TCS2−/− cells attenuates the hyperphosphorylation of ribosomal protein S6. LAMD-SM, ELT3, and ERC15 cells were microinjected with pEGFP and pEGFP-TSC2 plasmids expressing GFP or GFP-tagged tuberin, respectively. As seen in Fig. 6A, expression of tuberin significantly attenuated the phosphorylation level of ribosomal protein S6 in LAMD-SM cells. Similar results were obtained by microinjecting pEGFP-TSC2 into TSC2−/− ELT3 and TSC2−/− ERC15 cells, whereas the phosphorylation of ribosomal protein S6 was not inhibited in cells microinjected only with control pEGFP plasmid (Fig. 6B).
Quantitative analysis of the microinjection experiments are summarized in Table I and Fig. 6B and demonstrate that expression of tuberin in LAMD-SM, ELT3, and ERC15 significantly decreased the phosphorylation level of ribosomal protein S6.

**p70S6K Is Constitutively Active in LAMD-SM and TSC2−/− Cells and Is Reversed by Tuberin Expression**—Because S6 phosphorylation is mediated by p70S6K, we next examined whether p70S6K was similarly activated in cells lacking tuberin. Immunoblot analysis of cell lysates with a phospho-specific antibody revealed that in LAMD-SM, TSC2−/− ELT3, and TSC2−/− ERC15 cells p70S6K is phosphorylated on residues Thr-389 and Thr-421/Ser-424, which are critical sites required for kinase activation. In contrast, these residues were not phosphorylated in TSC2+/+ ASM and VSM cells (Fig. 7A). The p70S6K activity in LAMD-SM and tuberin-deficient cells was also markedly increased compared with levels found in TSC2+/+ ASM and VSM cells (Fig. 7B). Analysis of cell lysates demonstrated that the protein levels of p70S6K were comparable in all cell lines (Fig. 7A), suggesting that high p70S6K activity in LAMD-SM and tuberin-deficient cells was not due to increased expression of the enzyme. Furthermore, we examined whether the constitutive activation of p70S6K was due to the autocrine production of insulin-like growth factor 1, since it has been suggested that this factor is produced by ELT3 cells (48). However, a neutralizing
antibody directed against insulin-like growth factor-I had little effect on p70S6K activation after 48 h of incubation (data not shown), indicating that the constitutive activation of p70S6K is not due to autocrine activation by insulin-like growth factor-I. Furthermore, other signaling pathways known to be activated by insulin-like growth factor-I were not enhanced (see "Discussion").

We next examined whether the constitutive activation of p70S6K in LAMD-SM and TSC2+/−/H11546 cells could be reversed by tuberin expression. As seen in Fig. 8A, re-expression of tuberin in LAMD-SM, ELT3, and ERC15 cells (Fig. 8A) significantly decreased p70S6K activity. Phosphorylation of residue Thr-389 (Fig. 8C) but not Thr-421/Ser-424 (data not shown) was also significantly attenuated in cells transfected with tuberin. Our data correlate with previously described regulation of p70S6K activation by phosphorylation in vivo (49) and suggests that although p70S6K activity depends on phosphorylation of Thr-421/Ser-424, tuberin regulates phosphorylation of Thr-389. It remains to be determined which signaling molecules are involved in regulation of Thr-421/Ser-424 phosphorylation, and it is possible that transient expression of tuberin is

| Cell line | Plasmid | Number of cells | Number of phospho-S6-positive cells | Percentage of phospho-S6-positive cells |
|-----------|---------|-----------------|-------------------------------------|----------------------------------------|
| LAMD-SM   | pEGFP   | 153             | 97                                  | 63.4                                   |
| LAMD-SM   | pEGFP-TSC2 | 184           | 137                                 | 74.4                                   |
| LAMD-SM   | pEGFP-TSC2 | 110           | 33                                  | 30                                     |
| ELT3      | pEGFP   | 165             | 154                                 | 93.3                                   |
| ELT3      | pEGFP-TSC2 | 168           | 156                                 | 92.9                                   |
| ERC15     | pEGFP   | 310             | 68                                  | 21.9                                   |
| ERC15     | pEGFP-TSC2 | 199           | 178                                 | 89.4                                   |
| ERC15     | pEGFP   | 88              | 76                                  | 86.4                                   |
| ERC15     | pEGFP-TSC2 | 161           | 23                                  | 14.3                                   |

**TABLE I** Effect of microinjection of pEGFP-TSC2 on phosphorylation of ribosomal protein S6
not sufficient for affecting their activity.

PI 3-Kinase, Akt, and p44/p42 MAPK Activity in LAMD-SM and TSC2--/-- Cells—To examine whether the constitutive activation of p70S6K is due to the activation of its upstream modulators such as PI 3-kinase and Akt (50, 51), studies were performed to examine enzyme activation in LAMD-SM, TSC2--/-- ELT3, and TSC2+/+ ASM. As seen in Fig. 9A, PI 3-kinase activity, measured in anti-phosphotyrosine, anti-p85 regulatory subunit, anti-p110α catalytic subunit, and anti-p110β catalytic subunit, was comparable in LAMD-SM, ELT3, and ASM cells. Microinjection of inhibitory anti-VPS34 antibody into TSC2--/-- ELT3 cells had negligible effect on the phosphorylation level of ribosomal protein S6 (Fig. 9B), suggesting that this class of PI 3-kinase is unlikely to act upstream of p70S6K and ribosomal protein S6. The activation of Akt was also examined with a phospho-specific Ser-473 Akt antibody, since phosphorylation of this site is critical for activation of Akt. As seen in Fig. 9C, the levels of Akt phosphorylation were also comparable in LAMD-SM, ELT3, and ASM cells. In parallel experiments, the activation of p42/p44 MAPK, which plays a critical well established role in regulating cell proliferation, was examined with a phospho-specific antibody; here too there was little difference between LAMD-SM, ELT3, and ASM cells (Fig. 9D). These data demonstrate that basal levels of PI 3-kinase, Akt, and p42/p44 MAPK activation are comparable in LAMD-SM, TSC2--/-- ELT3, and TSC2+/+ ASM cells. In separate studies platelet-derived growth factor-induced activation of PI 3-kinase, Akt, and MAPK were also no different in TSC2--/-- or TSC2+/+ cell lines (data not shown). Collectively, these data also suggest that the constitutive activation of p70S6K and the hyperphosphorylation of S6 in ELT3 and LAMD-SM are specific and not due to PI 3-kinase or Akt activation.

DNA Synthesis in LAMD-SM and TSC2--/-- Cells—Because LAM disease is characterized by the increased proliferation of smooth muscle cells, we compared basal DNA synthesis levels in LAMD-SM, TSC2--/-- ELT3, and TSC2--/-- ERC15 to those levels observed in TSC2+/+ ASM and VSM cells. The mitotic index of serum-starved LAMD-SM, ELT3, and ERC15 cells, defined as the percentage of BrdUrd-positive nuclei compared with the total number of cells, was significantly higher than that of ASM and VSM cells (Fig. 10A). Expression of tuberin in LAMD-SM, ELT3, and ERC15 cells significantly decreased DNA synthesis (Fig. 10B) while having no effect on the mitotic index of tuberin-positive cells (data not shown).

Rapamycin Modulates p70S6K Activity and DNA Synthesis in LAMD-SM Cells—Because abnormal smooth muscle cell growth characterizes LAM lung disease, we next examined whether rapamycin, an inhibitor of p70S6K (52), modulates DNA synthesis in LAMD-SM cells. As seen in Fig. 11, rapamycin significantly inhibited DNA synthesis in LAMD-SM compared with cells treated with diluent alone. Similar effects of rapamycin were observed in ELT3 cells (data not shown). Over the duration of this assay, cell viability was unaffected by rapamycin, as determined by trypan blue staining and CaspATag fluorescein caspase activity assay (data not shown). These data suggest that p70S6K activation is critical for the proliferation of LAMD-SM cells.

DISCUSSION

Alterations in the function of tuberin, a product of the tumor suppressor gene TSC2, promote the development of benign tumors. This study shows that loss or mutation of tuberin activates p70S6K, leading to the hyperphosphorylation of ribosomal protein S6, which is necessary to stimulate the protein synthesis for progression through the cell cycle. In parallel PI 3-kinase, Akt, and MAPK, three other signaling molecules implicated in proliferation were unaffected by the TSC2 status of the cell. Importantly, we demonstrate that in LAM nodules the p70S6K/S6 pathway is activated. Furthermore, in primary cultures derived from these nodules, p70S6K and ribosomal protein S6 are also constitutively activated, and basal proliferative rates are elevated. Collectively, these data suggest that loss of tuberin may stimulate...
Our results identify tuberin as a novel regulator of p70S6K. Multiple studies reveal that p70S6K activation occurs through multisite phosphorylation mediated by multiple signaling pathways (52, 53). One well-established regulator is PI 3-kinase, which in part functions by activating the phosphoinositide-dependent kinase-1, which directly phosphorylates p70S6K on Thr-229 (54, 55). Another key regulator of p70S6K is mTOR, a critical regulator of protein synthesis that is involved in tuberin signaling. Interestingly, in normal human airway and vascular smooth muscle cells, rapamycin inhibits only mitogen-induced DNA synthesis but does not modulate the basal level of cell proliferation (37, 41), whereas in LAMD-SM and TSC2−/− cells, rapamycin abrogates abnormally high basal DNA synthesis. This critical difference may offer a potential therapeutic target to treat disease characterized by tuberin dysfunction. However, the role of mTOR in tuberin-dependent p70S6K activation remains to be elucidated. Phosphorylation of residues Thr-421/Ser-424 are also essential for p70S6K activation, although it has not previously been linked to the p70S6K pathway. Alternatively, Cdc42 and Rac1 have also been shown to regulate p70S6K, although the mechanism by which they function is not known (57, 58). Our results add further complexity to the activation of p70S6K.

As mentioned, tuberin encodes a GAP domain for both Rap1 (20, 59) and Rab5 (21) at its C terminus, raising the interesting possibility that these GTPases may also play a role in p70S6K activation. Although Rap1 has been well studied for its role in signaling (60), it has not previously been linked to the p70S6K pathway. Alternatively, Rab5 and Rap1 have been shown to function in vesicular trafficking, which may modulate p70S6K activity by indirectly altering its trafficking properties. It remains to be determined whether the GAP activity of tuberin is necessary or sufficient for activation of p70S6K signaling pathway and whether small GTPases may potentially contribute to these effects.

In the present study, we used two cell models to demonstrate that there are functional similarities and differences between LAMD-SM cells, which have TSC2 mutations, and TSC2−/− cells, which have a complete loss of TSC2. Both types of cells show constitutive activation of p70S6K, hyperphosphorylation of ribosomal protein S6, and increased proliferation rates. However, the magnitude of the effects is more profound in TSC2−/− cells, and expression of tuberin had greater inhibitory effect on
p70S6K activation and ribosomal protein S6 phosphorylation in TSC2−/− cells compared with LAMD-SM cells. It remains to be determined how specific alterations in the TSC2 gene are linked to the dysfunction of the cellular signaling pathway(s) that promotes abnormal cell growth and tumor development. Furthermore, the expression of tuberin in LAMD-SM and TSC2−/− cells attenuated but did not abrogate p70S6K activation and ribosomal protein S6 phosphorylation. Interestingly, expression of TSC2 significantly attenuated but did not completely inhibit DNA synthesis in LAMD-SM and TSC2−/− cells. This was largely due to limitations in transfection efficiency, since we measured the mitotic index of all cells, which includes both transfected and untransfected cells. Nevertheless, it is possible that other signaling molecules can be involved in p70S6K activation and DNA synthesis in these cells.

An important question arising from our study is, Does tuberin directly modulate p70S6K and S6 activation? Studies in Drosophila suggest a potential link between TSC2 gene and S6 kinase function (14). Comparison of mice with targeted disruption of TSC2 or S6K shows that although the tumor suppressor TSC2 gene is indispensable for embryonic development (15, 16, 61), loss of heterozygosity of the TSC2 gene leads to tumor development (7, 15, 16), and targeted disruption of S6K or conditional deletion of S6 appears critical for cell size determination and cell growth (27, 62). The prevailing thought suggests that p70S6K and ribosomal protein S6 regulate cell growth and proliferation and places p70S6K at a critical point in tumor development. The scope of our study was restricted to establishing the link between tuberin and p70S6K as it relates to LAM disease, whereas there are data indicating the potential role of p70S6K in other tumors (63, 64). The p70S6K activation, however, is likely only one signaling event affected by the loss of tuberin function, and tuberin-dependent tumor development possibly involves other unidentified molecular signals.

Histopathological changes in LAM are characterized by the development of tumor nodules, which consist of proliferating smooth muscle cells throughout the lungs. According to our data suggest that tuberin regulates p70S6K activation, there may exist a link between tuberin, p70S6K activation, and cell growth. Our results further suggest that tumors characterized by dysfunction of TSC2 may be amenable to therapies that abrogate constitutively active p70S6K. Further studies are necessary to determine the mechanism(s) by which tuberin suppresses p70S6K and ribosomal protein S6 activation.

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REFERENCES

1. Taylor, J. R., Ryu, J., Colby, T. V., and Raffin, T. A. (1990) N. Engl. J. Med. 323, 1254–1260.
2. Chu, S. C., Horiba, K., Usuki, J., Avila, N. A., Chen, C. C., Travis, W. D., Ferrans, V. J., and Moss, J. (1999) J. Clin. Invest. 104, 687–695.
3. Kobayashi, E., Minowa, O., Kuno, J., Minitani, H., Hino, O., and Noda, T. (1999) Cancer Res. 59, 1206–1211.
4. Yeung, R. X., Gao, J., Jin, F., Lee, W., Testa, J., and Knudson, A. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 11418–11424.
5. Kobayashi, T., Nishizawa, M., Hirayama, Y., Kobayashi, E., and Hino, O. (1995) Nucleic Acids Res. 23, 2608–2613.
6. European Chromosome 16 Tuberous Sclerosis Consortium (1993) Cell 75, 1305–1315.
7. Wienecke, R., König, A., and DeChue, J. E. (1995) J. Biol. Chem. 270, 16495–16441.
8. Xiao, G. H., Shairinejad, F., Jin, F., Golemis, E. A., and Yeung, R. S. (1997) J. Biol. Chem. 272, 6097–6100.
9. Henry, R. W., Yuan, X., Koszewski, N. J., Onda, H., Kwiatkowski, D. J., and Noonan, D. J. (1998) J. Biol. Chem. 273, 20355–20359.
10. Yu, J., Astrinidis, A., and Henske, E. P. (2001) Am. J. Genet. Med. Genet. 45, 63–80.
11. Matsuzato, Y., Horiba, K., Usuki, J., Chu, S. C., Ferrans, V. J., and Moss, J. (1999) Am. J. Respir. Cell. Mol. Biol. 21, 327–336.
12. Howe, S. R., Gottardis, M. M., Everitt, J. L., Wolf, D. C., and Walker, C. L. (1995) Am. J. Pathol. 146, 1568–1579.
13. Hino, O., Klein-Saanto, A. J. P., Freed, J. J., Testa, J. R., Brown, D. J., Henske, M. R., Tartof, K. D., and Knudson, A. G. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 327–331.
14. Walker, C. L., and Gimpl, J. (1992) Carcinogenesis 13, 25–32.
15. Panettieri, R. A., DePalma, L. R., Murray, R. K., Yadvich, P. A., and Kotlikoff, M. I. (1989) Am. J. Physiol. 256, C329–C335.
16. Gonnadorova, E., Ammit, A. J., Frizzi, C., Carroll, R. G., Eszterhas, A. J., Panettieri, R. A., and Krymskaya, V. P. (2001) Am. J. Physiol. Lung Cell. Mol. Physiol. 281, L1345–L1363.
17. Siddhanta, U., McIlroy, J., Shah, A., Zhang, Y., and Backer, J. M. (1998) Cancer Res. 58, 539–544.
18. Krymskaya, V. P., Hoffman, R., Eszterhas, A., Ciocca, V., and Panettieri, R. A. (1997) Am. J. Physiol. Lung Cell. Mol. Physiol. 273, C329–C34.
19. Panettieri, R. A., and Krymskaya, V. P. (2001) Am. J. Physiol. Lung Cell. Mol. Physiol. 281, L363–L375.
20. Kiguchi, K., Landes, G., Harris, P., and Walker, C. (2001) Am. J. Respir. Crit. Care Med. 164, 539–544.
57. Romanelli, A., Martin, K. A., Toker, A., and Blenis, J. (1999) Mol. Cell. Biol. 19, 2921–2928
58. Chou, M. M., and Blenis, J. (1996) Cell 85, 573–583
59. Wienecke, R., Maize, J. C., Shoarinejad, F., Vass, W. C., Reed, J. C., Bonifacino, J. S., Resau, J. H., Gunzburg, J. d., Yeung, R. S., and DeClue, J. E. (1996) Oncogene 13, 913–923
60. Bos, J. L., de Rooij, J., and Reedquist, K. A. (2001) Nat. Rev. Mol. Cell Biol. 2, 369–377
61. Rennebeck, G., Kleymenova, E. V., Anderson, R., Yeung, R. S., Artzt, K., and Walker, C. L. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 15629–15634
62. Shima, H., Pende, M., Chen, Y., Fumagalli, S., Thomas, G., and Rozga, S. C. (1998) EMBO J. 18, 6649–6659
63. Ostrowski, J., Woszczynski, M., Kowalczyk, P., Wocial, T., Hennig, E., Trzeciak, L., Janik, P., and Bomsztyk, K. (2000) Br. J. Cancer 82, 1041–1050
64. Seufferlein, T., and Rozengurt, E. (1996) Cancer Res. 56, 3895–3897
Tuberin Regulates p70 S6 Kinase Activation and Ribosomal Protein S6 Phosphorylation: A ROLE FOR THE TSC2 TUMOR SUPPRESSOR GENE IN PULMONARY LYMPHANGIOLEIOMYOMATOSIS (LAM)

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