Abnormal Growth of Smooth Muscle–Like Cells in Lymphangioleiomyomatosis

Role for Tumor Suppressor TSC2

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The TSC1 and TSC2 proteins, which function as a TSC1/TSC2 tumor suppressor complex, are associated with lymphangioleiomyomatosis (LAM), a genetic disorder characterized by the abnormal growth of smooth muscle–like cells in the lungs. The precise molecular mechanisms that modulate LAM cell growth remain unknown. We demonstrate that TSC2 regulates LAM cell growth. Cells dissociated from LAM nodules from the lungs of five different patients with LAM have constitutively activated S6K1, hyperphosphorylated ribosomal protein S6, activated Erk, and increased DNA synthesis compared with normal cells from the same patients. These effects were augmented by PDGF stimulation. Akt activity was unchanged in LAM cells. Rapamycin, a specific S6K1 inhibitor, abolished increased LAM cell growth. The full-length TSC2 was necessary for inhibition of S6 hyperphosphorylation and DNA synthesis in LAM cells, as demonstrated by co-microinjection of the C-terminus, which contains the GTPase activating protein homology domain, and the N-terminus, which binds TSC1. Our data demonstrate that increased LAM cell growth is associated with constitutive S6K1 activation, which is extinguishable by TSC2 expression. Loss of TSC2 GAP activity or disruption of the TSC1/TSC2 complex dysregulates S6K1 activation, which leads to abnormal cell proliferation associated with LAM disease.

Keywords: interstitial lung disease; smooth muscle; TSC

Lymphangioleiomyomatosis (LAM) is a rare lung disorder characterized by the abnormal growth of smooth muscle–like cells within the lung, which promotes cystic destruction of the lung and leads to loss of pulmonary function (1–3). Loss-of-function mutations in the tumor suppressor genes *tuberous sclerosis complex 1* (*TSC1*) and *TSC2* are associated with pulmonary LAM (4–7). The *TSC1* and *TSC2* genes encode two proteins, TSC1 and TSC2, also known as hamartin and tuberin, respectively, which function as a TSC1/TSC2 complex (8). LAM is associated predominantly with *TSC2* mutations, indicating that TSC2 function is critical for sustaining normal cell function (5). Despite considerable research efforts in defining the role of TSC2 in cell proliferation and its relevance to LAM pathobiology, the cellular mechanisms that modulate LAM cell growth remain unknown.

TSC2, a 200-kD, ubiquitously expressed, evolutionary conserved protein, contains in its C-terminus region a GTPase-activating protein (GAP) homology domain that functions as a GAP for the small GTPase Rheb (Ras homologue enriched in brain) (9–12). It has been reported that TSC2 can function as a GAP for small GTPase Rab5, which is critical for vesicular trafficking (13). The functional link between TSC2 and Rab5 remains to be established. TSC2 inhibits Rheb activity by reducing levels of GTP-Rheb, which leads to the suppression of the mammalian target of the rapamycin (mTOR)/S6 kinase 1 (S6K1) signaling pathway (9, 10, 14–17). In the presence of growth factors and abundant nutrients, TSC2 activity is suppressed, leading to increased Rheb and mTOR/S6K1 activity. Studies using established TSC2-deficient cell lines or TSC2 overexpression show that the GAP activity of TSC2 is critical for regulating the mTOR/S6K1 pathway. Rheb overexpression induces mTOR/S6K1 activation in HEK293 cells (10, 15), and TSC2, which has a mutation in the GAP domain, is unable to inhibit Rheb activity and Rheb-dependent activation of S6K1 in COS-7 and HEK293E cells (9, 16). Although Rheb is a physiologic substrate for TSC2 GAP activity, it is not well understood whether the GAP function of TSC2 is necessary and sufficient for inhibiting mTOR/S6K1 activity and cell proliferation in LAM.

TSC2, through its N-terminus region, binds TSC1, and both proteins form the TSC1/TSC2 complex (8, 18–20). Some studies demonstrated that TSC1 and TSC2 are required for maximal GAP activity toward Rheb (9, 11, 16). Other studies have shown that TSC2 alone is sufficient to promote Rheb activation (10, 12). We demonstrated that deletion of the putative TSC1-binding domain of TSC2 attenuates the growth inhibitory effect of TSC2 re-expression in TSC2-deficient ELT3 cells (21). It is poorly understood whether TSC1 contributes to the GAP activity of TSC2 on Rheb.

Although TSC2 may function as a GAP for Rheb, which may contribute to cell proliferation, the role that TSC2 and its GAP domain play in modulating LAM cell growth remains undefined. Using molecular approaches, we show that the C-terminus of TSC2 (amino acids 1114–1784), containing the GAP domain, alone is not sufficient to inhibit ribosomal protein S6 hyperphosphorylation and increased proliferation of LAM cells. Similarly, the N-terminus of TSC2 (amino acids 1–1113), containing the TSC1-binding domain, had little effect on S6 hyperphosphorylation and DNA synthesis. However, co-microinjection of the N-terminus and the C-terminus comprising the full-length TSC2 inhibited the hyperphosphorylation of ribosomal protein S6 and DNA synthesis in LAM cells. Our study indicates that the C-terminus and the N-terminus domain are critical for the negative regulation of mTOR/S6K1 activity and inhibition of LAM cell proliferation.

MATERIALS AND METHODS

Cell Cultures

LAM cells were dissociated from LAM nodules dissected from the lungs of patients with LAM who had undergone lung transplant (22).

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Briefly, tissue was subjected to an enzymatic digestion in M199 medium containing 0.2 mM $CaCl_2$, 2 mg/ml collagenase D (Roche, Indianapolis, IN), 1 mg/ml trypsin inhibitor (Sigma Chemical Co., St. Louis, MO), and 3 mg/ml elastase (Worthington, Lakewood, NJ). The cell suspension was filtered and washed with equal volumes of cold DF8 medium consisting of equal amounts of Ham's F12 and DMEM supplemented with 1.6×10^{-6} M ferrous sulfate, 1.2×10^{-5} U/ml vasopressin, 1.0×10^{-9} M tri-iodothyronine, 0.025 mg/ml insulin, 1.0×10^{-8} M cholesterol, 2.0×10^{-7} M hydrocortisone, 10 pg/ml transferrin, and 10% FBS. Aliquots of the cell suspension were plated at a density of 1.0×10^4 cells/cm^2 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. LAM cells in subculture during the third through twelfth cell passages were used. LAM-1/1, LAM-2/4, LAM-2/7, LAM-2/8, and LAM-2/9 cells carry *TSC2* gene mutations and have no immunoreactivity to HMB45 (22). The list of LAM cells used in this study is presented in the Table 1. Because there is no established LAM cell nomenclature, we labeled LAM cells in the order in which tissue was acquired from the LAM registry; this corresponds to the first digit in the LAM cell number (Table 1). The second digit corresponds to the cell population derived from the LAM tissue of one patient. For example, the LAM-1/1 represents the total population of cells derived from the LAM nodule of patient number 1; LAM-2/4, LAM-2/7, LAM-2/8, and LAM-2/9 denote cells from the second patient where the tissue was split into different cell populations that were derived and grown separately. In these studies, we used populations 4, 7, 8, and 9. Human bronchus fibroblasts (HBFs), used as a control cells, were dissociated from the bronchus of the same patient with LAM according to the protocol used for LAM cell dissociation. LAM and bronchus tissues were obtained in compliance with the University of Pennsylvania Institutional Review Board approved protocol and the protocol approved by the LAM Registry at the National Heart, Lung, and Blood Institute.

Human airway smooth muscle (ASM) cells, human pulmonary arterial vascular smooth muscle (HVSM) cells, and human lung fibroblasts (HLFs), which were also used as control normal mesenchymal cells, were dissociated and maintained as previously described (22, 23). TSC2-deficient ELT3 and ERC15 cell lines were derived and maintained as previously described (24, 25). All assays were performed on cells maintained in serum-free medium for 48 h.

Transient Transfection and Microinjection

Transient transfection with plasmids, which were prepared using the EndoFree Plasmid Maxi Kit (Qiagen Inc., Valencia, CA), was performed using the Effectene transfection reagent (Qiagen Inc., Valencia, CA) according to the manufacturer's protocol. Microinjection was performed using an Eppendorf Microinjection System (Hamburg, Germany) (22). Briefly, cells were plated on 2-well glass chamber slides (Nalgene Nunc International, Naperville, IL) and transfected or microinjected with pEGFP, pEGFP-N-TSC2, encoding 1–1113 amino acids of TSC2; pEGFP-C-TSC2, encoding 1114–1784 amino acids of TSC2; or co-microinjected with pEGFP-N-TSC2 and pEGFP-C-TSC2 followed by immunocytochemical analysis or 5-bromo-2′deoxyuridine (BrdU) incorporation assay. Immunoblot analysis of GFP-tagged TSC2 constructs was previously described (21).

TABLE 1. LAM CELLS AND HBFs USED FOR EXPERIMENTS

LAM patient number	LAM cells	Normal HBFs from the same patients
1	LAM-1/1	–
2	LAM-2/4	–
	LAM-2/7	
	LAM-2/8	
	LAM-2/9	
3	LAM-3/12	–
4	LAM-4/29	HBF-031202
5	LAM-5/52	HBF-041902

Immunocytochemistry

Cells were fixed with 3.7% paraformaldehyde (Polysciences, Inc., Warrington, PA), permiabilized with 0.1% Triton X-100 (Sigma Chemical Co.), and blocked as previously described (21). Anti-phospho-ribosomal protein S6 (S235) antibody (Upstate Biotechnology, Lake Placid, NY) was used at a 1:50 dilution, anti-GFP rabbit serum (Molecular Probes, Eugene, OR) was used at a 1:200 dilution, and secondary antibody Alexa Fluor 594 donkey anti-sheep IgG conjugate was used at a 1:400 dilution; Alexa Fluor 488 goat anti-rabbit IgG conjugate (Molecular Probes) was used at a 1:400 dilution, and anti-smooth muscle α-actin clone 1A4 FITC-conjugated antibody (Sigma Chemical Co.) was used at a 1:200 dilution. Cells were visualized using a Nikon Eclipse TE2000-E or a Nikon Eclipse E400 microscope (Nikon, Melville, NY) under appropriate filters.

Cell Proliferation Assay

DNA synthesis was assessed by BrdU incorporation assay as described previously (22). Briefly, serum-deprived cells were incubated with BrdU for 24 h followed by immunocytochemical analysis with 2 μg/ml primary mouse anti-BrdU antibody (Becton Dickinson, San Jose, CA) and then with 10 μg/ml secondary Texas Red–conjugated anti-mouse antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). The cells were examined using a fluorescent microscope Nikon Eclipse E400 under ×200 magnification. The mitotic index was defined as the percentage of BrdU-positive nuclei per field per the total number of cells per field. A total of ~ 200 cells were counted per each condition in each experiment.

S6K1 Activity Assay

S6K1 was precipitated from cell lysates with anti-S6K1 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Immunocomplexes were collected by protein A-Sepharose (Pharmacia, Biotech AB, Uppsala, Sweden) followed by an *in vitro* kinase activity assay in the presence of S6K/Rsk2 substrate peptide (KKRNRTLTK) (Upstate Biotechnology) and [γ-^{32}P] ATP (NEN Dupont, Boston, MA). The reaction was stopped by spotting the reaction mixture onto p81 phosphocellulose filters. The radioactivity of samples was measured using a Beckman LS 6500 scintillation counter (22, 26).

Immunoblot Analysis

Serum-deprived cells were lysed in S6K1 or RIPA lysis buffer, and equal amounts of lysate, adjusted to protein content, were subjected to immunoblot analysis with anti-S6K1, anti–phospho-Thr389 S6K1, anti-phospho-Thr421/Ser424 S6K1, anti-ribosomal protein S6, anti–phospho-ribosomal protein S6 (Ser235/236), anti-Akt, anti-phospho-Akt (Ser473), anti-p44/p42 MAP kinase, or anti–phospho-p44/p42 MAP kinase (Thr202/Tyr204) antibodies (Cell Signaling Technology, Inc., Beverly, MA) as previously described (22). Image analysis was performed using the Gel-Pro analyzer program (Media Cybernetics, Silver Spring, MD).

Data Analysis

Data points from individual assays represent the mean values ± SE. Statistically significant differences among groups were assessed with the ANOVA (Bonferroni-Dunn), 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

Primary LAM Cells Maintain Smooth Muscle α-Actin Expression

Because LAM nodules consist of smooth muscle α-actin–positive cells, we examined whether primary cultures of LAM cells express smooth muscle α-actin in cell culture. LAM cells are spindle-shaped, grow in parallel arrays, and form a distinctive "hills-and-valleys" pattern (Figure 1). Five primary LAM cell cultures derived from the lung of five different LAM patients (*see* Table 1) stained positive for smooth muscle α-actin (Figures 1B–F); these levels were comparable to the levels of smooth muscle α-actin

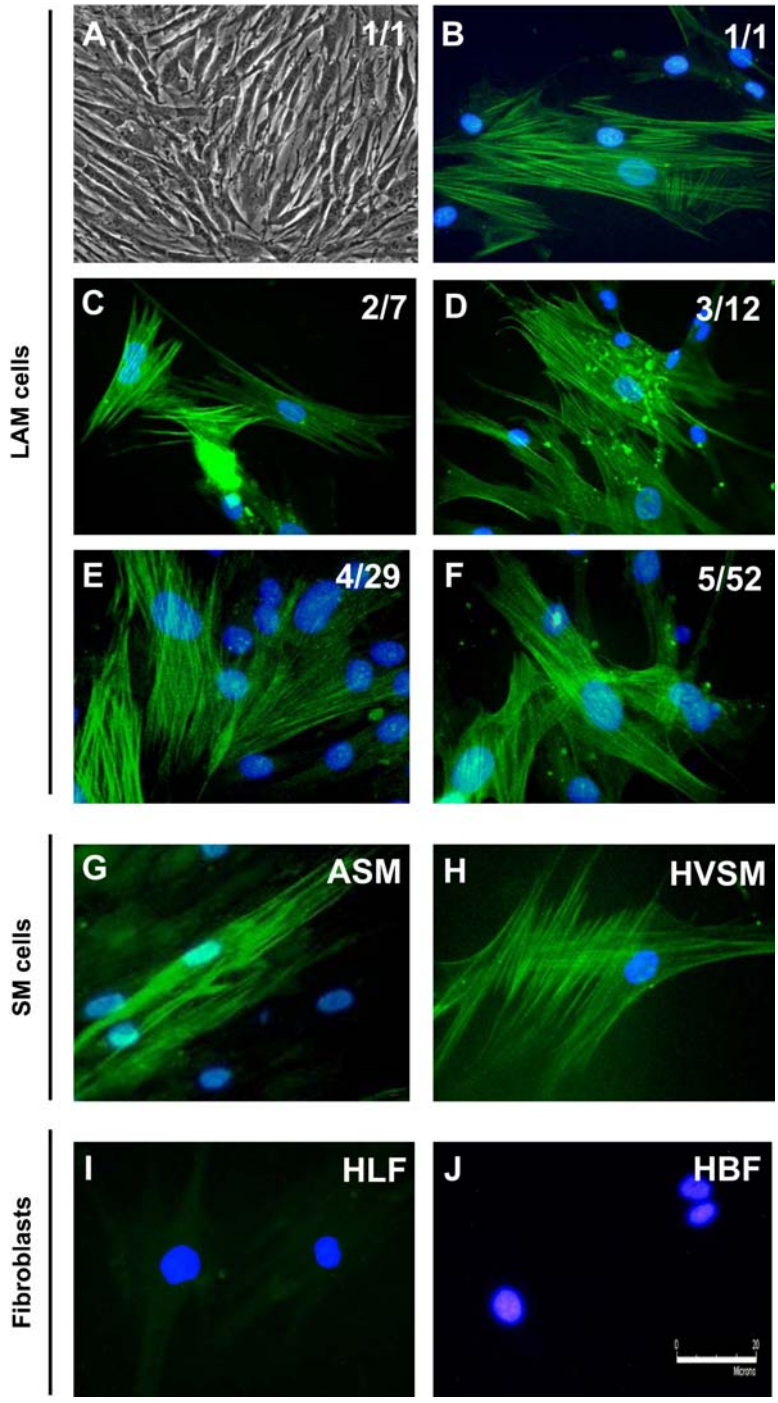


Figure 1. (*A*) LAM cell morphology. LAM cells show typical smooth muscle–like cell morphology. The phase contrast image shown is representative of five LAM cell cultures derived from different patients with LAM. Image was taken on an Olympus IX microscope (*original magnification*: ×200). (*B–J*) Smooth muscle α-actin expression. Serum-deprived LAM-1/1, LAM-2/7, LAM-3/12, LAM-4/29, LAM-5/52, ASM cells, HVSM cells, HLFs, and HBFs were subjected to immunocytochemical analysis with anti-smooth muscle α-actin FITC-conjugated antibody (SM α-actin, *green*) followed by incubation with 4,6-diamidino-2-phenylindole to detect nuclei (*blue*). Images were taken using a Nikon Eclipse TE2000-E microscope (*original magnification*: ×400) and are representative of three separate experiments.

found in primary ASM and HVSM cell cultures (Figures 1G and 1H). In contrast, HBFs, dissociated from the normal bronchus of the same patients with LAM and normal HLFs, were negative for smooth muscle α-actin (Figures 1I and 1J). These data show that primary LAM cells in culture express smooth muscle α-actin, suggesting that these cells retained a smooth-muscle–like phenotype after dissociation from LAM nodules.

LAM Cells Have Increased DNA Synthesis

Because LAM disease is characterized by the abnormal proliferation of smooth muscle-like cells within the lung (1, 2), we examined proliferation levels of LAM cells dissociated from the lung of five different patients with LAM. All five serum-deprived LAM cell cultures had increased DNA synthesis compared with control normal HBFs, HLFs, and HVSM cells (Figure 2A). PDGF stimulation at the concentration that is known to stimulate human smooth muscle cell proliferation (27) further enhanced DNA synthesis in LAM cells compared with diluent-treated cells. PDGF-induced proliferation was markedly higher for all LAM cells compared with control HBFs, HLFs, and HVSM cells, which indicates that growth factors may augment high proliferation rates of LAM cells (Figure 2B). These data show that primary LAM cell cultures have increased DNA synthesis and PDGF further augments LAM cell proliferation.

Because rapamycin, a specific inhibitor of mTOR and a known inhibitor of cell growth (28), has a differential effect on

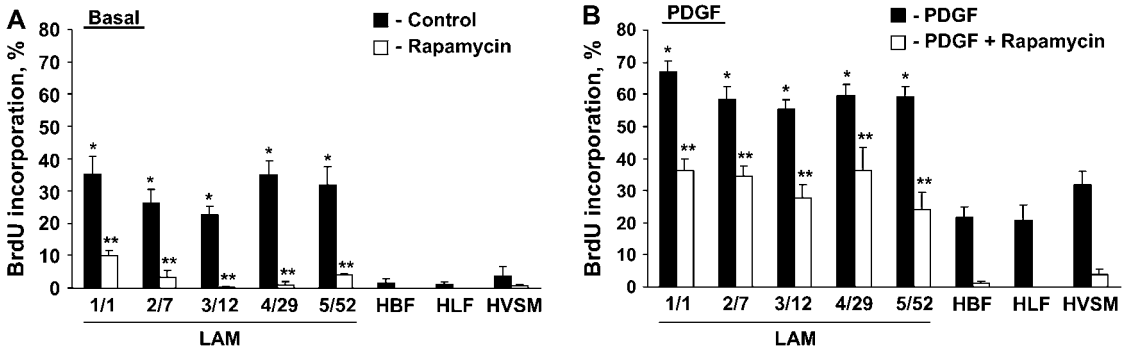


Figure 2. Primary LAM cells have increased DNA synthesis. LAM-1/1, LAM-2/7, LAM-3/12, LAM-4/29, LAM-5/52, HVSM cells, HBFs, and HLFs were serum deprived for 48 h and pretreated for 30 min with 200 nM rapamycin or diluent. Cells were left unstimulated (Basal) (*A*) or were stimulated (*B*) with 10 ng/ml PDGF for 16 h. BrdU was added, and 24 h later, DNA synthesis was assessed by immunohistochemical analysis of BrdU incorporation as described in MATERIALS AND METHODS. BrdU incorporation represents the percentage of BrdU-positive cells compared with the total number of cells. Data represent means ± SE of three separate experiments. *$P < 0.001$ for LAM versus HBFs, HLFs, and HVSM. **$P < 0.001$ for rapamycin-treated cells versus diluent-treated cells by ANOVA (Bonferroni Dunn).

the inhibition of cell proliferation depending on cell type (27, 29–31), we examined whether proliferation of LAM cells derived from different patients is modulated by rapamycin. We have published data that show that rapamycin inhibits LAM-1/1 cell proliferation in a concentration-dependent manner (22). Rapamycin significantly inhibited basal and PDGF-induced DNA synthesis in all analyzed LAM cells (Figures 2A and 2B). Although rapamycin abrogated DNA synthesis of serum-deprived LAM cells, it attenuated only PDGF-induced DNA synthesis, in contrast to the complete inhibition found in HBFs, HLFs, and HVSM cells (Figure 2B). PDGF, a potent mitogen, activates the phosphatidylinositol 3-kinase (PI3K)/S6K1 and ERK pathways (27), which are essential for cell cycle progression and proliferation. Rapamycin abrogates PDGF-induced proliferation in normal HBFs, HLFs, and HVSM cells (Figure 2B). In contrast, in LAM cells the effect of rapamycin is partial, which is ~ 30% inhibition. These data indicate that PDGF may contribute to the differential sensitivity of LAM cells to rapamycin, which might be an important consideration in the therapeutic treatment of LAM with rapamycin.

mTOR/S6K1 Is Constitutively Activated in LAM Cells

Because TSC2 function is associated with the modulation of the mTOR/S6K1 signaling pathway (22, 32, 33) and with the constitutive hyperphosphorylation of ribosomal protein S6, a hallmark of *TSC2* deficiency and loss of TSC2 function, we examined S6 phosphorylation in five primary LAM cells. Immunocytochemical analysis demonstrated an increased phosphorylation of S6 in all five primary LAM cell lines compared with ASM cells (Figure 3A). LAM cells are primary cultures that show some degree of heterogeneity (22). The fraction of phospho-S6–positive cells in the examined LAM cell lines was 76–92% of the total number of cells, depending on cell line; all smooth muscle α-actin–positive LAM cells were also phospho-S6 positive. Only stimulation of ASM cells with PDGF induced S6 phosphorylation comparable with phospho-S6 levels in LAM cells.

Because mTOR is an obligated upstream activator of S6, we examined whether rapamycin affects S6 phosphorylation. ASM cells, stimulated with PDGF, were used as a positive control. Rapamycin inhibited ribosomal protein S6 hyperphosphorylation in serum-deprived LAM-1/1 cells and in PDGF-stimulated ASM cells (Figure 3A). Immunoblot analysis with phospho-S6 antibody further demonstrated that in serum-deprived LAM cells, ribosomal protein S6 was markedly phosphorylated compared with control ASM cells and HBFs (Figure 3B). Treatment with PDGF markedly increased S6 phosphorylation in control HBFs and ASM cells but had little effect on the phospho-S6 level in LAM cells. Quantitative analysis of three separate experiments shows that ribosomal protein S6 is constitutively hyperphosphorylated in serum-deprived LAM cells compared with ASM cells and HBFs, and PDGF significantly promoted S6 phosphorylation in control HBFs and ASM cells but not in LAM cells. The level of PDGF-induced S6 phosphorylation in ASM cells and HBFs was markedly lower than basal S6 phosphorylation in LAM cells (Figure 3C), demonstrating that S6 is hyperphosphorylated in the absence of stimuli in LAM cells. Together, these data show that primary LAM cells have hyperphosphorylated ribosomal protein S6, which is sensitive to rapamycin.

Because S6 phosphorylation is regulated by S6K1, we examined whether S6K1 was activated in primary LAM cell cultures derived from different patients with LAM. Immunoblot analysis of cell lysates revealed that in all LAM cells, S6K1 is phosphorylated on the Thr421/Ser424 and Thr389 residues, which is critical for its activation. In contrast, these sites were not phosphorylated in ASM and HVSM cells that were used as controls (Figure 4A). Analysis of S6K1 activity in the same cells confirmed that primary LAM cell lines have markedly increased S6K1 activity compared with levels detected in ASM and HVSM cells (Figure 4B). These data demonstrate that primary LAM cell cultures are characterized by the constitutive activation of the S6K1/S6 pathway, which is the molecular signature of TSC2 loss of function.

Akt Activity in LAM Cells

Because Akt is a major upstream modulator of the mTOR/S6K1 signaling pathway (34), we examined whether this signaling protein is activated in LAM cells. Analysis of Akt activation was performed with a phospho-Ser-473 Akt antibody, the phosphorylation of which is critical for Akt activation. The immunoblot analysis detected two Akt isoforms in LAM cells and HBFs, with slight differences in phosphorylation levels depending of cell type. However, the total levels of Akt phosphorylation had some, but not marked, differences in serum-deprived LAM cells, ASM cells, and HBFs (Figure 5A, *top panel*). PDGF-stimulated Akt phosphorylation was also comparable in LAM cells, ASM cells, and HBFs (Figure 5A, *middle panel*). These data show that loss of TSC2 function in LAM cells does not affect Akt signaling and that the constitutive activation of mTOR/S6K1 is independent from Akt activity; this correlates with data obtained from other cell types (33).

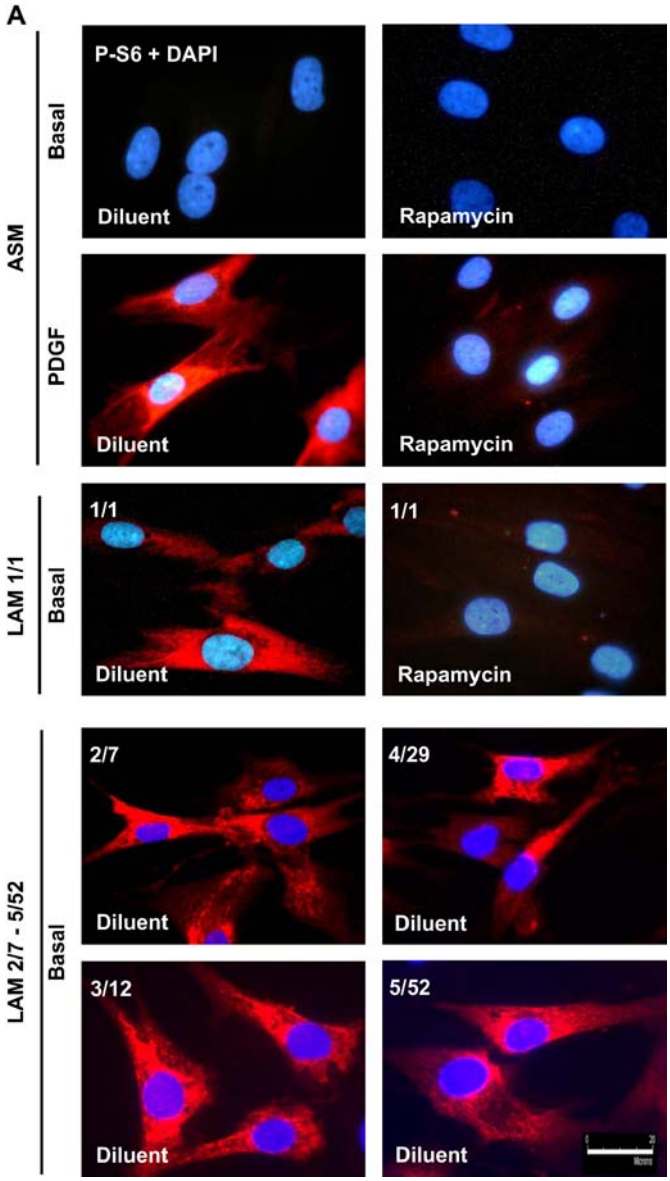


Figure 3. (*A*) Rapamycin inhibits S6 phosphorylation in LAM cells. LAM-1/1, LAM-2/7, LAM-3/12, LAM-4/29, LAM-5/52, and ASM cells were serum deprived, and LAM-1/1 cells were preincubated with 200 nM rapamycin or diluent for 30 min. Control ASM cells were treated with rapamycin followed by stimulation with 10 ng/ml of PDGF. Cells were fixed, and immunocytochemical analysis with anti-phospho-ribosomal protein S6 antibody (P-S6, *red*) was performed followed by incubation with 4,6-diamidino-2-phenylindole (*blue*). Images were taken using a Nikon Eclipse E400 microscope (*original magnification*: ×400) and are representative of three separate experiments. (*B*) Ribosomal protein S6 is hyperphosphorylated in primary LAM cells. Serum-deprived LAM-1/1, LAM-2/7, LAM-3/12, LAM-4/29, LAM-5/52, ASM cells, and HBFs were stimulated with 10 ng/ml PDGF or diluent and subjected to immunoblot analysis with phospho-ribosomal protein S6 (P-S6) and S6 antibodies. Images are representative of three separate experiments. (*C*) Quantitative analysis of S6 phosphorylation. Phosphorylation levels of S6 were calculated using Gel-Pro analyzer software and normalized to total S6 protein levels. P-S6 in ASM cells was taken as a one fold. Data are means ± SE of three separate experiments. *$P < 0.01$ for LAM cells versus ASM cells and HBFs. **$P < 0.01$ for nonstimulated cells versus PDGF-stimulated cells by ANOVA (Bonferroni Dunn).

ERK Is Constitutively Activated in LAM Cells

Because it was shown that ERK can act upstream of TSC1/TSC2 complex and may play a critical role in the phosphorylation and inactivation of TSC2 (1, 35, 36), we examined the activation of ERK in five different LAM cell cultures with phospho-specific antibody. Our data demonstrate that in serum-deprived LAM cells, phosphorylation of ERK is higher compared with ASM cells and HBFs (Figure 5B, *top panel*). PDGF promotes ERK phosphorylation in LAM cells, control ASM cells, and HLFs to comparable levels (Figure 5B, *middle panel*). These data demonstrate that ERK is constitutively phosphorylated in LAM cells in the absence of any stimuli and suggest that ERK-dependent signaling may contribute to LAM disease progression.

Regulation of Ribosomal Protein S6 Phosphorylation Requires the N-Terminus and the C-Terminus of TSC2

TSC2 regulates the Rheb/mTOR/S6K1 signaling pathway through its GAP domain located in its C-terminus (9, 10, 14). Evidence suggests that TSC2 forms a complex with TSC1 through the TSC1-binding domain (1–418 amino acids) located in its N-terminus region (18, 19). We examined whether TSC2 alone is required for modulating mTOR/S6K1 activity or if binding with TSC1 is necessary for TSC2 GAP function. Because ribosomal protein S6 is activated in all LAM cells, we choose the LAM-1/1 cell line for examining this molecular approach. We used DNA constructs encoding GFP-tagged TSC2 regions: the N-terminus (1–1113 amino acids), containing the TSC1-binding domain, and the C-terminus (1114–1784 amino acids), which includes the GAP domain (1517–1674 amino acids) (21). Our data show that only TSC2 constructs containing the TSC1-binding domain N-TSC2 and HBD-TSC2 form a complex and can be co-immunoprecipitated with TSC1 (Figure 6).

LAM cells were microinjected with plasmids, expressing the GFP-tagged, full-length TSC2, N-terminus of TSC2, the C-terminus of TSC2, or control GFP or co-microinjected with the N- and C-terminuses of TSC2 followed by immunocytochemical analysis with anti-phospho S6 antibody. The co-microinjection technique was used to provide direct delivery of equal amounts of C- and N-terminuses TSC2 constructs to the cells. Expression of the N- or the C-terminus of TSC2 alone had little effect on S6

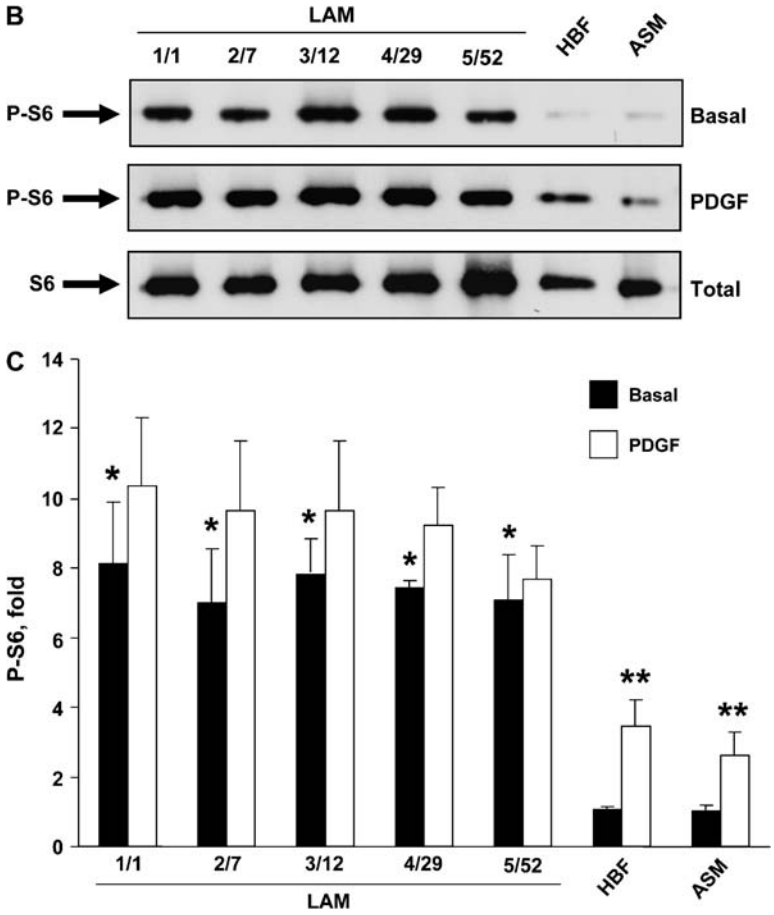


Figure 3. Continued

phosphorylation and was comparable to S6 phosphorylation in cells expressing control GFP (immunofluorescent images are not shown; statistical analysis is presented in Figure 7C). Only co-microinjection of the N- and C-terminuses of TSC2 significantly inhibited S6 phosphorylation (Figure 7A). Similar results were obtained by microinjecting TSC2-deficient ELT3 and ERC15 cells (Figure 7B). Quantitative analysis of the microinjection experiments demonstrates that expression of full-length TSC2 or the C- and N-terminus domains comprising full-length TSC2, but not the C- or N-terminus of TSC2 alone, markedly decreased the phosphorylation level of ribosomal protein S6 in LAM cells (Figure 7C). Similar data were obtained for TSC2-deficient ELT3 and ERC15 cells: Co-injection of N- and C-terminuses of TSC2 decreased S6 phosphorylation by 49.4 ± 2.4% and 56.7 × 3.7%, respectively. These data demonstrate that the C-terminus of TSC2 containing the GAP domain is necessary but not sufficient for regulating S6 phosphorylation and suggest that full-length TSC2 is required for the inhibition of constitutive S6 hyperphosphorylation in LAM cells.

The N- and C-Terminuses of TSC2 Are Required for Inhibition of LAM Cell Proliferation

Because full-length TSC2 is required for inhibition of ribosomal protein S6 hyperphosphorylation, which is critical for cell proliferation, we examined whether these effects correlate with the N- and C-terminus of TSC2 expression on DNA synthesis in LAM cells. LAM, ELT3, and ERC15 cells were microinjected with plasmids expressing the GFP-tagged N-terminus of TSC2, the C-terminus of TSC2, or control GFP or co-microinjected with the N- and C-terminuses of TSC2 followed by BrdU incorporation assay. The N-terminus and C-terminus of TSC2 alone had little effect on LAM cell proliferation, but co-expression of the N- and C-terminals of TSC2 significantly inhibited DNA synthesis by 45.1 ± 6.7%, 49.2 ± 1.2%, and 43.3 ± 3.7% in LAM-1/1, ELT3, and ERC15 cells, respectively (Figure 7D). These data demonstrate that the N- and C-terminals comprising the full-length TSC2 are required for inhibiting LAM and TSC2-deficient cell proliferation.

DISCUSSION

LAM is characterized by the abnormal growth of smooth muscle–like cells within the lung (1–3), which is associated with loss of function of tumor suppressor proteins TSC1 and TSC2 (4–7). In this study, we show that five different primary LAM cell cultures dissociated from the LAM nodules from the lungs of five different patients with LAM have increased DNA synthesis, hyperphosphorylated ribosomal protein S6, and constitutively activated ERK in the absence of any stimuli and that these effects were further augmented by PDGF stimulation. We demonstrate that the C-terminus of TSC2 is necessary but not sufficient for regulating S6 phosphorylation and DNA synthesis in human primary LAM cells. The N-terminus region of TSC2 is also necessary but not sufficient for these functions of TSC2, suggesting that TSC1/TSC2 interaction may be required for the GAP activity of TSC2. Our study indicates that full-length TSC2 and both the C- and N-terminus domains are able to provide optimal regulation of ribosomal protein S6 activation and DNA synthesis in LAM and TSC2-deficient cells.

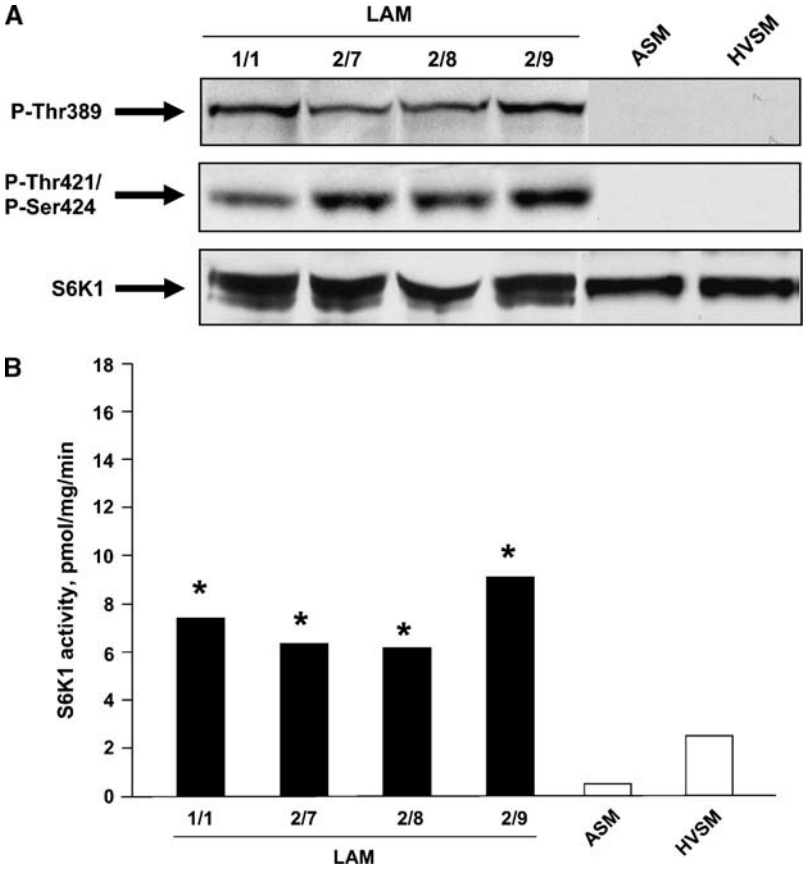


Figure 4. S6K1 is constitutively active in primary LAM cells. (*A*) LAM-1/1, LAM-2/7, LAM-2/8, LAM-2/9, ASM, and HVSM cells were serum deprived, and cell lysates, equal in protein content, were probed with anti–phospho-Thr389 S6K1 (P-Thr389), phospho-Thr421/Ser424 S6K1 (P-Thr421/P-Ser424), or S6K1 antibodies. Images are representative of three separate experiments. (*B*) *In vitro* S6K1 activity was measured in serum-deprived LAM cells, ASM cells, and HVSM cells. $*P < 0.001$ for LAM cells versus ASM and HVSM cells by ANOVA (Bonferroni Dunn).

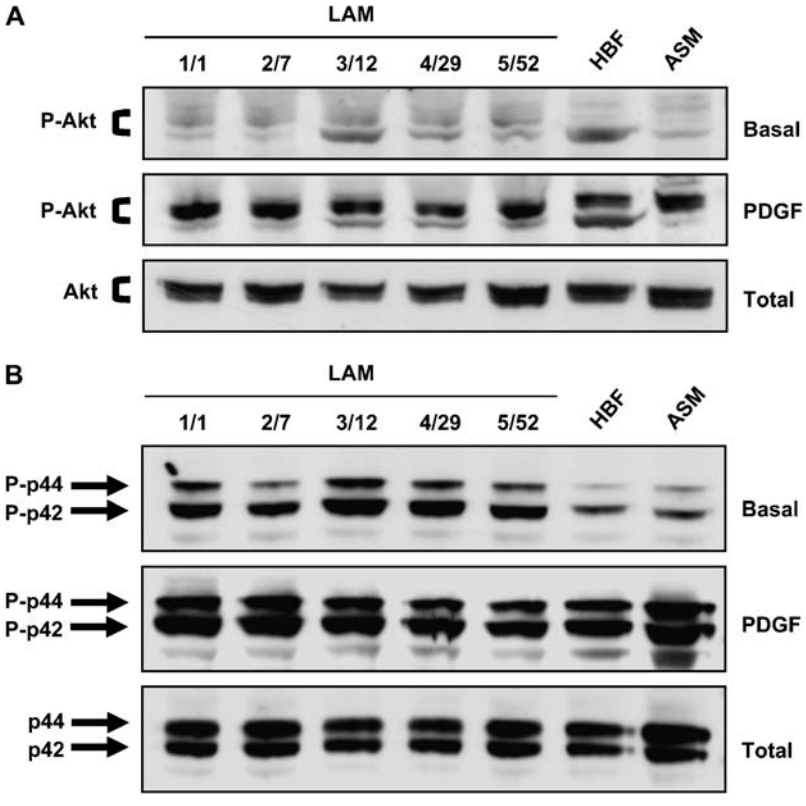


Figure 5. Akt and ERK activity in LAM cells, ASM cells, and HBFs. Serum-deprived LAM-1/1, LAM-2/7, LAM-3/12, LAM-4/29, LAM-5/52 cells, ASM cells, and HBFs were stimulated with 10 ng/ml PDGF or diluent, and immunoblot analysis of cell lysates, equal in protein content, was performed with phospho-Akt (Ser-473) (P-Akt), Akt (*A*), anti-p44/p42 MAP kinase, or anti–phospho-p44/p42 MAP kinase (Thr202/Tyr204) (*B*) antibodies. Images are representative of three independent experiments.

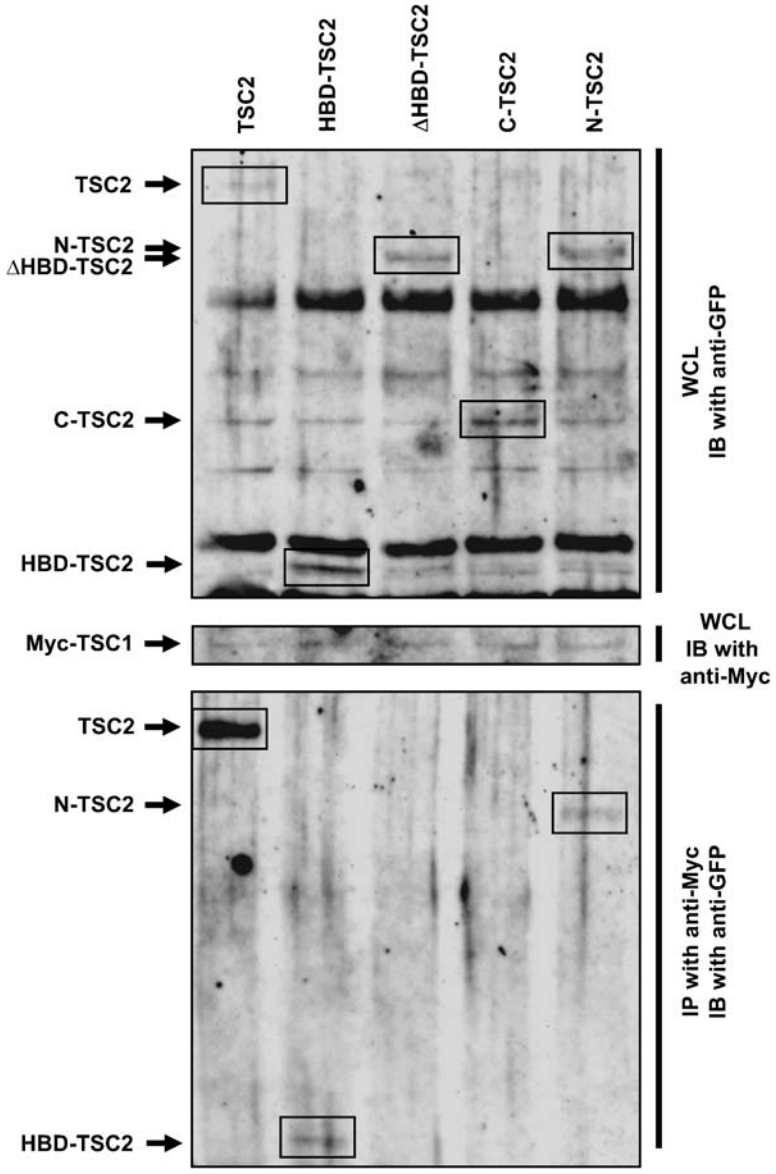
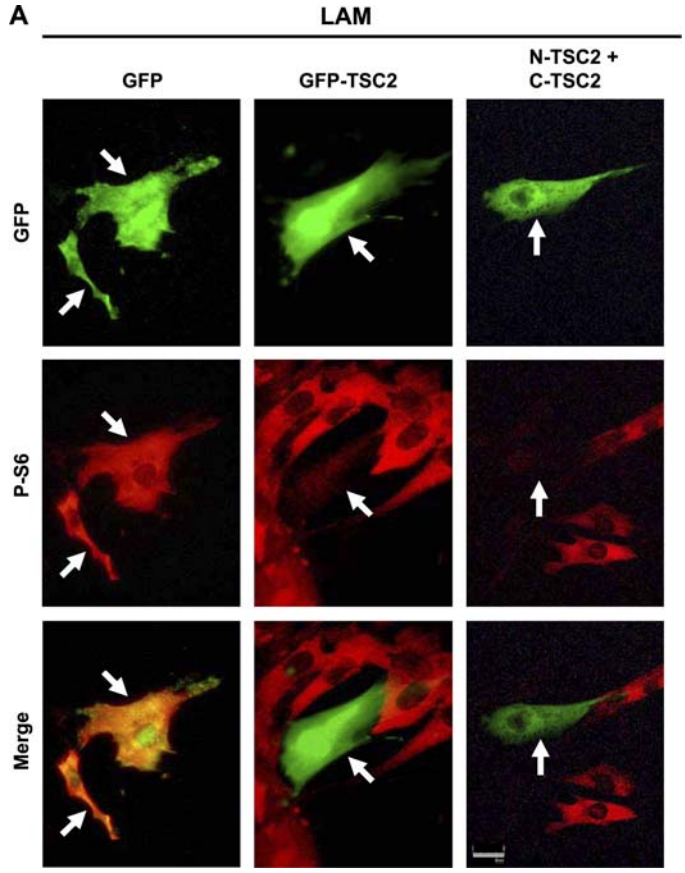


Figure 6. Co-immunoprecipitation of TSC2 constructs with TSC1. Serum-deprived ELT3 cells were co-transfected with Myc-tagged TSC1 and GFP-tagged TSC2 constructs: full-length TSC2, TSC1-binding domain of TSC2 (HBD-TSC2) (1–460 amino acids of TSC2), TSC2 without TSC1-binding domain (ΔHBD-TSC2) (461–1784 amino acids of TSC2), C-TSC2 (1114–1784 amino acids of TSC2), or N-TSC2 (1–1113 amino acids of TSC2). Coexpression of TSC2 constructs and Myc-TSC1 was detected with anti-GFP (*top panel*) and anti-Myc antibodies (*middle panel*), respectively. Immunoprecipitation with anti-Myc antibody was performed, followed by immunoblot analysis of immunoprecipitates with anti-GFP antibody to detect GFP-tagged TSC2 constructs co-precipitated with Myc-tagged TSC1 (*bottom panel*). Images are representative of two separate experiments. *Rectangles* indicate GFP-tagged TSC2 constructs.

LAM can occur as an isolated disorder (sporadic LAM) or in association with TSC, an autosomal disorder affecting 1 in 6,000 individuals who develop hamartomas and benign tumors in the brain, heart, and kidney (37, 38). LAM pathology is characterized by abnormal cell proliferation due to TSC1 and TSC2 loss of function, but disease severity is predominantly associated with the loss of TSC2 (4, 5). Genetic studies of patients with TSC also show that TSC is associated with *TSC1* or *TSC2* mutations (39–42); however, patients with *TSC2* mutations had a greater degree of disease severity compared with those with *TSC1* mutations (40–42). Additionally, loss of TSC2 resulted in abnormal cell proliferation, which was reversed by rapamycin treatment, in TSC2−/− MEFs, ELT3, and ERC15 cells (22, 43). Our data demonstrate that primary LAM cell cultures derived from the lungs of five different patients have increased DNA synthesis without any stimuli, which was inhibited by rapamycin; these results extend our previous findings (22). PDGF further increased the high proliferation rate of LAM cells. The effect of rapamycin on PDGF-stimulated LAM cell proliferation was partial, indicating that mTOR/S6K1-independent mechanisms may be involved in the PDGF-dependent LAM cell proliferation, which might be important for the treatment of LAM disease with rapamycin. Evidence suggests that PDGF activates the PI3K/S6K1 and ERK pathways (27), which are critical for cell proliferation. Our data show that ERK is upregulated in LAM cells and that PDGF further promotes ERK activation. Additionally, inhibition of ERK leads to partial inhibition of PDGF-dependent LAM cell proliferation (our unpublished observation). Taken together, these data suggest that mTOR/S6K1 and ERK signaling pathways may contribute to LAM disease progression, and further studies are needed to establish a role of ERK signaling in PDGF-dependent LAM proliferation. TSC2 suppresses cell growth by regulating mTOR/S6K1 activation (8, 38, 44). Our data show that LAM cell cultures have constitutively active S6K1 and hyperphosphorylated S6 in the absence of any stimuli, which is similar to what was found in TSC2-deficient MEFs, ELT3, and ERC15 cells (22, 43).

TSC2 suppresses cell growth and mTOR/S6K1 activation by functioning as a GTPase-activating protein (10, 38, 44). Recent genetic and biochemical studies identified small GTPase Rheb as a direct target of TSC2 GAP activity (9, 10, 14, 38). However, the function of TSC2 in physiologically relevant cell types, such



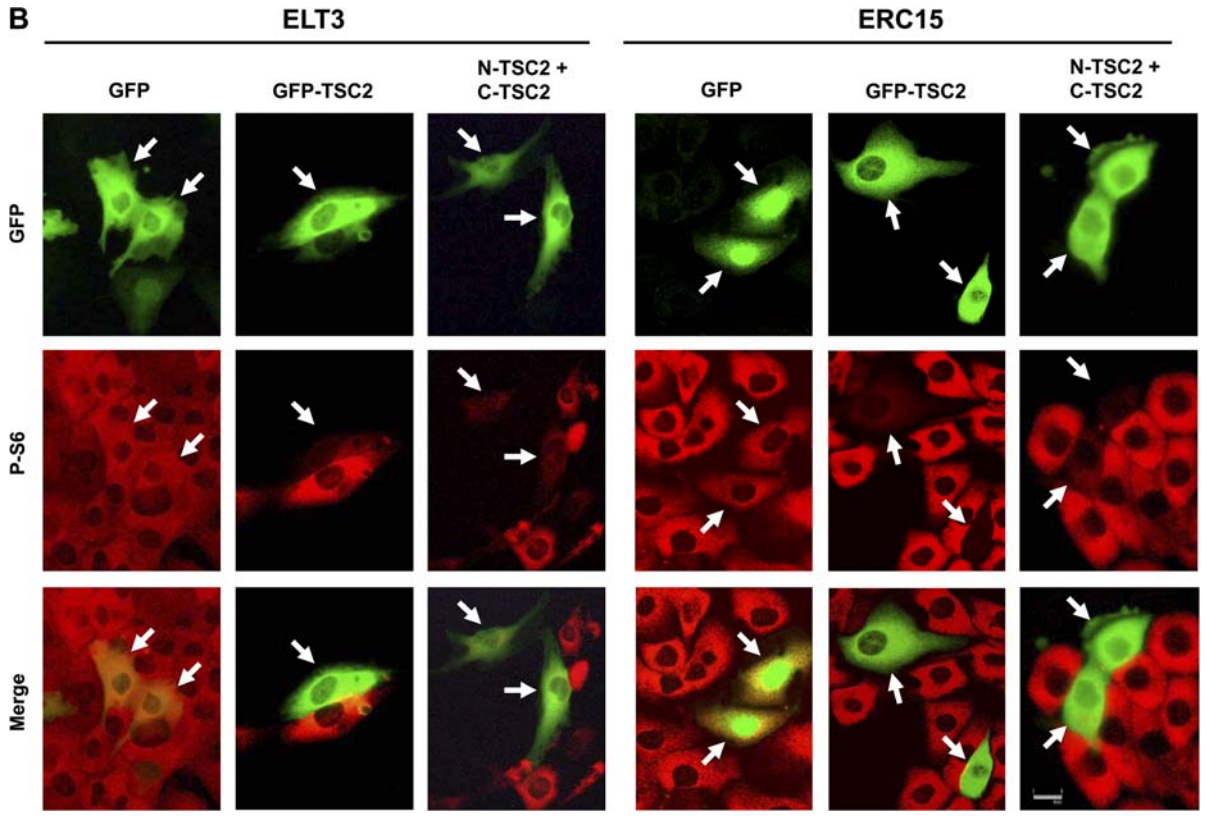


Figure 7. Inhibition of ribosomal protein S6 phosphorylation and cell proliferation require the N- and C-termini of TSC2. Serum-deprived LAM-1/1 (*A*), ELT3, and ERC15 cells (*B*) were microinjected with GFP, GFP-TSC2, or co-microinjected with GFP–N-TSC2 and GFP–C-TSC2 followed by immunostaining with anti-phospho-ribosomal protein S6 (P-S6, *red*) and anti-GFP antibodies (*green*); yellow fluorescence results from the co-localization of P-S6 and GFP in cells. Images were analyzed on a Nikon Eclipse E400 microscope (*original magnification*: ×400) and are representative of three separate experiments. *Arrows* indicate microinjected cells. (*C*) Quantitative analysis of microinjection experiments. Data represent the percentage of P-S6–positive cells per total number of microinjected cells; S6 phosphorylation of GFP-microinjected cells was taken as 100%. Data are means ± SE of three separate experiments. $*P < 0.001$ for GFP-TSC2 or N-TSC2 + C-TSC2 versus GFP by ANOVA (Bonferroni Dunn). (*D*) Serum-deprived LAM-1/1, ELT3, and ERC15 cells were microinjected with plasmids expressing GFP as a control, GFP–N-TSC2, or GFP–C-TSC2 or co-microinjected with GFP–N-TSC2 and GFP–C-TSC2 followed by BrdU incorporation assay. BrdU incorporation represents the percentage of BrdU-positive cells expressing GFP-tagged constructs compared with the total number of transfected cells. BrdU incorporation of GFP-microinjected cells was taken as 100%. Data represent means ± SE of three separate experiments. $*P < 0.001$ for N-TSC2 + C-TSC2 versus GFP by ANOVA (Bonferroni Dunn).

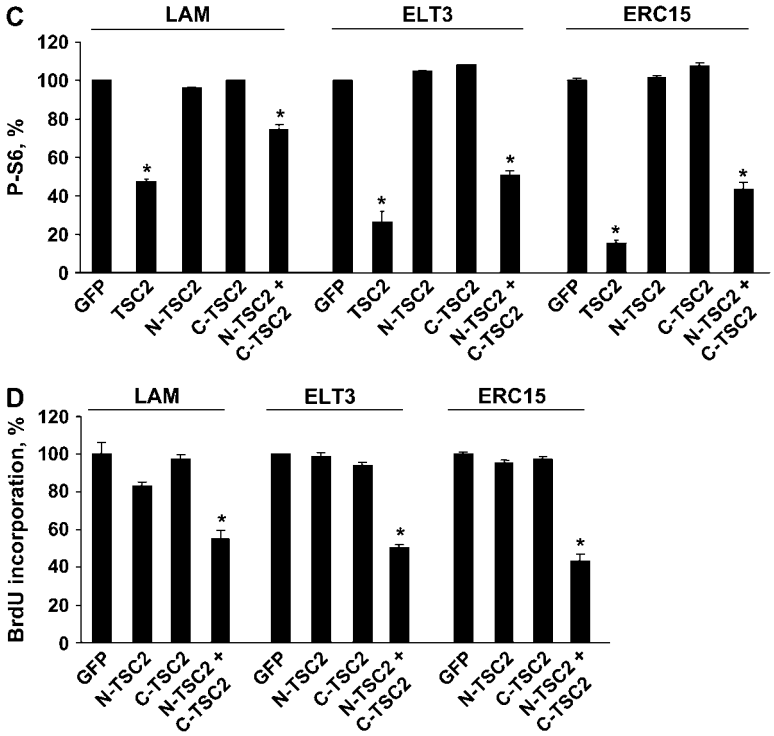


Figure 7. Continued

as human LAM cells, remains to be determined. In our study, we show that the C-terminus of TSC2, amino acids 1114–1784, is necessary but not sufficient for inhibiting ribosomal protein S6 phosphorylation and DNA synthesis. The stable expression of the larger truncated mutant of the C-terminus of TSC2, amino acids 1049–1809, in Eker tumor cell lines LExF2 and ERC18M partially decreased colony formation and showed partial morphologic reversion compared with full-length TSC2 (45). TSC2 constructs coding the C-terminus region, including the GAP domain, exhibited suppressed tumorigenicity in the Eker rat (45, 46). Our published studies also demonstrate that expression of the larger C-terminus TSC2 mutant partially inhibits S6 phosphorylation and DNA synthesis (21). Collectively, these data indicate that other regions containing, for example, Akt, AMPK, or MAPK phosphorylation sites, are required for the GAP function of TSC2.

Evidence suggests that Akt functions as an upstream modulator of growth factor-induced mTOR/S6K1 activation (34). Akt through phosphorylation of TSC2 releases mTOR/S6K1 from TSC2-dependent inhibition (33). Our data show that in primary LAM cell cultures, Akt is not activated in the absence of any stimuli and that PDGF-stimulated Akt phosphorylation is comparable to levels found in ASM cells and HBFs. These data correlate with our published studies demonstrating that basal levels of Akt phosphorylation were comparable in LAM, TSC2−/− ELT3, and ASM cells (22) and that PDGF promoted a marked activation of PI3K in the TSC2−/− ELT3 cells (47). Additionally, it was shown that loss of TSC2 in TSC2−/− MEFs inhibits activation of Akt by IRS-dependent growth factor pathways, such us insulin or IGF-I, but has little effect on IRS-independent PDGF-induced Akt activation in TSC2−/− MEFs (48). In contrast, Zhang and colleagues demonstrated that PDGF receptor (PDGFR) α and PDGFR β are downregulated in the TSC2−/− MEFs, which led to a reduction of PI3K/Akt activation by PDGF, insulin, and serum (43). We found that PDGFR β is expressed in TSC2−/− rat ELT3 cells and is activated upon PDGF stimulation; furthermore, the re-expression of TSC2 in these cells had little effect on PDGFR β levels, suggesting that PDGF-induced signaling is not defective at the receptor level (21).

TSC2 functions in a complex with TSC1, the loss of which leads to mTOR/S6K1 activation and cell growth (39). TSC2 binds TSC1 through its specific binding domain located in its N-terminus region (18, 49, 50); the N-terminus of TSC2 and TSC1 have no GAP activity toward Rheb (10–12). However, a number of studies on mammalian cell lines indicated that TSC1/TSC2 interaction is required for TSC2 function as a regulator of the Rheb/mTOR/S6K1 signaling pathway (9–11, 16). Inoki and colleagues demonstrated that the GAP activity of TSC2 requires the full-length protein because neither the N-terminus nor the C-terminus region, which contains the GAP domain, displayed GAP activity toward Rheb in HEK293 cells (10). Tee and colleagues showed that overexpression of TSC2 or TSC1 in HEK293 cells enhanced the intrinsic GTPase activity of Rheb by $\sim$ 2-fold, but co-expression of TSC2 and TSC1 enhanced Rheb GTPase activity by $>$ 100-fold over the activity of either alone (16). Garami and colleagues reported that TSC1 alone had no effect on the amount of GTP-bound Rheb but found strongly potentiated GAP activity of TSC2 in COS cells (9). Nellist and colleagues demonstrated that some missense mutations outside the TSC2 GAP domain completely inactivated the TSC1/TSC2 complex, inhibited TSC2 GAP activity, and promoted the phosphorylation of the downstream effectors of mTOR in MEFs (20). Because the binding of TSC1 with TSC2 prevents TSC2 ubiquitination and degradation (50, 51), it is possible that TSC1 promotes the GAP function of TSC2 by its stabilization. Our data show that the N-terminus region of TSC2 alone has little effect on S6 phosphorylation and DNA synthesis, but its presence is necessary for the inhibition of S6 phosphorylation and DNA synthesis in human LAM and TSC2-deficient ELT3 and ERC15 cells. Taken together, these data indicate the

importance of TSC1/TSC2 interaction for the function of TSC2 as a negative regulator of the Rheb/mTOR/S6K1 pathway. Our data also demonstrate that the GAP function of TSC2 is necessary for the inhibition of cell proliferation: The N-terminus of TSC2 without the GAP domain is unable to inhibit DNA synthesis in human LAM and TSC2-deficient rat cells; however, co-microinjection of the N- and C-terminuses comprising full-length TSC2 inhibits LAM cell proliferation.

In summary, our current findings provide evidence that the C-terminus of TSC2 is necessary, but not sufficient, for regulating S6 activity and DNA synthesis in primary human LAM cells and in TSC2-deficient ELT3 and ERC15 cells. The C- and N-fragments, containing the GAP domain and the TSC1-binding domain, respectively, are involved in ribosomal protein S6 and DNA synthesis inhibition, suggesting that the GAP activity of TSC2 and TSC1/TSC2 interaction are required for TSC2 to function as a tumor suppressor. Because the loss of TSC2 or TSC1 functions is associated with LAM disease (4–7), the loss of TSC2 GAP activity or the loss of the ability TSC2 and TSC1 to form a complex may de-regulate GAP-dependent regulation of the Rheb/mTOR/S6K1 signaling pathway and LAM cell growth, which could be key events in the pathobiology of LAM.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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