Estrogen-induced Smooth Muscle Cell Growth Is Regulated by Tuberin and Associated with Altered Activation of Platelet-derived Growth Factor Receptor-β and ERK-1/2*

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The mechanisms that regulate the diverse responses to estrogen (E2) are unknown. Loss of function of the tuberous sclerosis 2 gene (TSC2), a tumor suppressor gene, has been associated with a growth-promoting effect of E2. We hypothesized that tuberin, the protein product of TSC2, binds to estrogen receptors (ER) and regulates the growth effect of E2. An in vivo association between full-length tuberin and ERα was observed in HEK 293 cells and ELT-3 smooth muscle cells. In contrast, poor association was observed between tuberin and ERβ. Complex formation with ERα and the C-terminal end of tuberin was also observed in vivo and in vitro, indicating that binding between ERα and tuberin occurs at the C-terminal end of the tuberin molecule. We examined the effect of tuberin expression in ELT-3 smooth muscle cells on the growth response to E2. The growth-promoting effect of E2 in tuberin-null ELT-3 smooth muscle cells was ERα-specific, associated with up-regulation and activation of platelet-derived growth factor receptor-β (PDGFRβ) and activation of the signaling intermediate, extracellular signal-regulated kinase-1/2 (ERK-1/2). In contrast, the expression of tuberin in ELT-3 smooth muscle cells resulted in significant abrogation of E2-stimulated growth. In parallel with this observation, the expression of tuberin in ELT-3 cells also resulted in significant inhibition of PDGFRβ and ERK-1/2 activation in response to E2. These results demonstrate that tuberin binds specifically to ERα and inhibits E2-induced proliferation of ELT-3 cells. Furthermore, the opposing effects of tuberin on estrogen-induced activation of PDGFRβ and ERK-1/2 suggest a pivotal role for tuberin in directing the signaling events that dictate the growth response to E2.

Estrogen is a naturally occurring steroid hormone that has widespread and diverse biologic actions (1). Estrogen is important for the normal growth and differentiation of many reproductive tissues and can also stimulate the growth of benign and malignant tumors of the breast and endometrium (2, 3). However, the study of cellular growth in response to estrogen has revealed both stimulatory and inhibitory properties of estrogen on vascular smooth muscle cells and vascular endothelial cells (4–8). Although much is known about the tissue-specific growth effects of estrogen, less is known about the mechanisms that underlie this diverse response.

The biological effects of estrogen were traditionally thought to be determined by estrogen receptors ERα and ERβ, where the main effect occurs through transit of the ligand-bound receptor to the nucleus and consequent transcriptional activation of ER (1, 9). The diverse and variable effects of estrogens can be explained by different ligand affinities and tissue distributions of ERα and ERβ, the large number of cytoplasmic chaperone proteins that regulate estrogen/ER complex location, and a large family of nuclear proteins (co-repressors and co-activators) that modulate transcription driven by estrogen response elements in the nuclear genome (10–12). In addition to the transcriptional or “genomic” effects of estrogen, recent reports have highlighted the importance of rapid signaling events that occur within the first 5–15 min of exposure to estrogen that are not associated with altered gene transcription (13, 14). Among the most well studied signaling targets of estrogen are members of the mitogen-activated protein (MAP) kinase family, extracellular signal-regulated kinases ERK-1 (p42 MAP kinase) and ERK-2 (p44 MAP kinase) (15–18). Studying the mechanisms that regulate the genomic and non-genomic effects of estrogen are key to understanding the diverse biological effects of estrogen.

Tuberin is a tumor suppressor protein that is encoded by the tuberous sclerosis complex 2 (TSC2) gene located on chromosome 16 (19). Patients with inactivation of one copy of the TSC2 gene develop tuberous sclerosis complex (TSC). TSC is characterized by benign tumor growths of the skin, heart, brain, kidney, and lungs. Abrupt expression of TSC2 (tuberin) occurs in the lungs and kidneys of patients with lymphangioleiomyomatosis, a disease that primarily affects females (19–21). Furthermore, loss of function of TSC2 is associated with

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** The abbreviations used are: ER, estrogen receptor; E2, 17β estradiol; MAP, mitogen-activated protein; ERK, extracellular signal-regulated kinase; PDGFR, platelet-derived growth factor receptor; PDGF, platelet-derived growth factor receptor; TSC, tuberous sclerosis complex; GFP, green fluorescence protein; mTOR, mammalian target of rapamycin; ELT, Eker rat leiomyoma tumor; HEK, human embryonic kidney; GST, glutathione S-transferase; THC, R,R-tetrahydrocannabinol.

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aberrant ER expression in proliferating smooth muscle cells found in the kidney and lung, suggesting that tuberin is involved in regulating the response of smooth muscle cells to estrogen (20, 22, 23). Although tuberin has not been shown to have direct transcriptional capacity, it has been shown that overexpression of tuberin in HEK 293 cells inhibits steroid- and ER-mediated transcription (24–26). It has also been shown that tuberin localizes to cytoplasmic and nuclear cellular compartments to bind to and modify the function of cellular proteins such as hamartin, calmodulin, and cyclins (26–28). Several lines of evidence now also confirm that tuberin is a phosphorylated protein, that phosphorylation is regulated by Akt, and that tuberin expression is associated with reduced phosphorylation of the ribosomal protein S6, which lies downstream of mTOR (mammalian target of rapamycin) (reviewed in Ref. 29). In addition, recent studies have also highlighted a novel relationship between tuberin and members of the MAP kinase family, ERK and p38 (30–32). Thus, it is now clear that tuberin is a protein that functions as a signaling molecule that can modulate many potential intracellular pathways involved in the regulation of cell growth. Previously, we analyzed the growth effects of estrogen in uterine-derived tuberin-null ELT-3 smooth muscle cells and tuberin-expressing vascular smooth muscle cells (33). In vascular smooth muscle cells that constitutively express tuberin, the growth inhibitory effect of estrogen was associated with down-regulation of PDGFRβ and PDGFRα and limited activation of ERK MAP kinase. In contrast, the positive growth effect of estrogen in tuberin-null ELT-3 cells was dependent upon the up-regulation of PDGFB and PDGFRβ and prolonged activation of ERK MAP kinase. Although the opposing effects of estrogen may have been cell type-specific, we hypothesized that the opposing effects of estrogen were due to the altered expression of tuberin. Herein, we demonstrate that the growth-stimulating effect of estrogen in tuberin-null ELT-3 smooth muscle cells is ERα-specific and is associated with the activation of PDGFRβ and ERK-1/2 MAP kinase. We show that tuberin binds specifically to ERα and inhibits estrogen-induced growth of ELT-3 cells, an effect that is associated with attenuated activation of PDGFRβ and ERK-1/2 MAP kinase. These results suggest that tuberin functions as a key protein in the regulation of estrogen-induced cellular growth and that tuberin acts as a molecular switch to dictate the growth response to estrogen in ELT-3 smooth muscle cells.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**

ELT-3 cells (Eker rat uterine leiomyoma-derived smooth muscle cells) were obtained from and characterized by Dr. Cheryl Walker (M. D. Anderson Cancer Center) (34). ELT-3 cells were grown and maintained in DF8 medium as previously described (34). The experiments were performed on ELT-3 cells between passages 29 and 32. Human embryonic kidney (HEK) 293 cells were cultured in Dulbecco’s modified Eagle medium supplemented with 10% fetal bovine serum. In all experiments using 17β estradiol (E2) (Sigma), serum was withdrawn for at least 24 h prior to the administration of E2 in serum-free medium.

**Plasmid Constructs**

Standard recombinant DNA technologies were utilized to develop mammalian expression constructs of TSC2 as previously described (20, 24, 26). The GST fusion constructs of TSC2 are described elsewhere (26).

**Adenovirus Production**

Recombinant adenovirus expressing various TSC2 cDNA constructs were created using the AdEasy vector system (36). Full-length TSC2 (Sall and XbaI), the C-terminal 672 amino acids of TSC2 (SalI and XbaI), a full-length TSC2 with a sequence for green fluorescence protein (GFP) fused to its C terminus (NheI and XbaI), and human ERα (EcoRI) were restriction-digested from their previously described mammalian expression constructs (24–26) and subcloned into an appropriately digested (SalI, XbaI; EcoRV, XbaI; or EcoRI) pShuttle vector. These constructs were subsequently used to transform electroporation-compotent AdEasy-1 BJ5183 cells (37) containing a copy of the pAdEasy vector. pAdEasy-pShuttle-TSC2 recombinants were identified by restriction analysis and authenticated by DNA sequencing through their recombination sites using vector-specific primers. pAdEasy-TSC2 recombinants were used in CaPO4 transfection (38) of 90% confluent HEK 293 cells and stored for plaque formation. The overexpression of tuberin for these various constructs was established by Western blotting and probing with anti-tuberin antisera (Santa Cruz Biotechnology) and/or anti-GFP antisera (Clontech). Viral cultures from appropriate expression constructs were amplified in HEK 293 cells and stored at −70 °C. Viral infections were generally carried out in experimental protocols by simply adding 100 μl of the amplified culture to a 90% confluent plate of test cells (HEK 293). The cells were incubated with virus for 18–24 h and processed as described below.

**Retrovirus Production**

Generation of retroviral vectors and revertant cells is described in brief. PT67, pLXIN, pIREShyg, and hygromycin B were purchased from Clontech. A DNA fragment containing IRES and hygromycin B phosphotransferase gene was PCR-amplified from pIREShyg3 and replaced the DNA sequence of IRES and the hygromycin resistant gene in pLXIN from BamHI and SphI sites. Human TSC2 CDNA was then subcloned into the Xhol and BamHI sites of the pIREShyg retroviral vector. The plasmids were transfected with LipofectAMINE 2000 (Invitrogen) into retroviral packing PT67 cells. Filtered medium containing retrovirus was stored until use.

Retrovirally mediated transfer of either TSC2 or empty vector was achieved by treating ELT-3 cells with equal amounts of retrovirus in the presence of DF-8 medium containing 10% serum. After 24 h, the virus was withdrawn, and the cells were grown for an additional 24 h in medium containing 10% serum. For the preparation of stable cell lines expressing TSC2, hygromycin (100 μg/ml) was used to select TSC2 expressing colonies of ELT-3 cells. Four individual colonies of TSC2 expressing ELT-3 cells were grown, passaged, and expanded. Protein lysates were prepared, and the expression of tuberin was assessed by Western immunoblot as described below.

**Assessment of Cell Growth**

**Coulter Counting**—The cells were seeded at a density of 0.5 × 10⁴ cells/ml on 35-mm Petri dishes. When cultures achieved 10–20% confluency, the serum was withdrawn from the culture medium for 24–48 h. The cell cultures were treated with 10 nM E2 (Sigma) in the absence of serum and were counted using a Coulter counter (Technical Communications, Coulter Electronics) at the intervals indicated in the text. All experiments were performed in quadruplicate and repeated at least twice to ensure reproducibility.

**³H/Thymidine Incorporation**—The protocol used has been described in detail (39). The cells were seeded at a density of 0.5 × 10⁴ cells/ml on 96-well plates. When cultures achieved 30–40% confluency, serum was withdrawn from the culture medium for 24 h. The cells were treated with 10 nM E2 (Sigma) in the absence of serum, and ³H/Thymidine (0.5 μCi/well; PerkinElmer Life Sciences) was added to the culture medium at the specified times indicated in the text. The cells were washed, and radioactivity was counted. The experiments were performed in quadruplicate and repeated at least twice to ensure reproducibility.

**Inhibitors**

Inhibitors used in these studies include the specific ER inhibitor ICI 182,780 (1 μM; Torcis, Ellenville, MO) and the selective ERβ inhibitor THC (R,R-tetrahydrochrysene, 10⁻⁶ M; kindly provided by Dr. Benita S. Katzenellenbogen, University of Illinois (40)). THC is a nonsteroidal ligand structurally unrelated to the typical ER antagonists, tamoxifen, and ICI 182,780. The R,R-enantiomer of THC is a pure ERβ antagonist with a relative binding affinity of 25% compared with E2. Other inhibitors used include actinomycin D (0.05 μg/ml; Calbiochem, La Jolla, CA) and rapamycin (10 μM; Cell Signaling Technology), which specifically inhibits the activation of mTOR and downstream phosphorylation of S6. The length of treatment and the doses are indicated in the text.

**Preparation of Cell Extracts**

The preparation of whole cell and membrane extracts has been previously described (33).
Immunoprecipitation experiments were performed as previously described (33). In brief, 5 μg of PDGFRβ polyclonal antibody (R & D Systems, Minneapolis, MN), tuberin (C terminus) polyclonal antibody, or ERα antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was incubated at 4 °C overnight with 0.5–1 mg of protein extracts. Protein A-Sepharose beads (Repligen, Cambridge, MA) were then added and rocked at 4 °C for an additional 2 h. The precipitated immune complexes were washed, and the reaction was terminated by the addition of 2× Laemmli SDS-PAGE sample buffer. The samples were boiled and resolved by SDS-PAGE. In all of the experiments, the membranes were subsequently stripped and reblotted with an appropriate antibody to ensure immunoprecipitation of the protein of interest. To maximize the detection of ERα in samples immunoprecipitated with anti-tuberin and to minimize contaminating signal from precipitated immunoglobulin, antisera of a different species was used.

**Protein Pull-down Assays**

GST and GST-TSC2-C672 fusion proteins were prepared from BL21-RP *Escherichia coli* cells essentially as previously described (26). Full-down assays were performed similar to previous studies (24). Briefly, glutathione-Sepharose beads (Amersham Biosciences) were washed and pre-equilibrated in Buffer A (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 10% glycerol, and 1% Nonidet P-40) to generate a 50% slurry. Approximately 30 μl of the 50% slurry was used per sample to bind 20 μg of purified GST-TSC2-C672. The protein-bead mixture was incubated at 4 °C for 30 min with rotation to allow binding. Unbound material was eliminated with repeated washes with Buffer A. The GST-TSC2-C672-Sepharose beads were subsequently incubated overnight at 4 °C with 1.25 μg of either recombinantly purified human ERα or human ERβ in the presence or absence of 17β estradiol in a total volume of 500 μl of Buffer 1. Unbound ERα or ERβ was removed by extensive washing with Buffer A. The beads were pelleted by centrifugation and re suspended in 20 μl of 2× SDS-electrophoresis buffer. The proteins were separated on a 10% SDS-PAGE gel, Western blotted to nitrocellulose filters, and probed with the indicated antisera. The blots were developed with ECL reagents (PerkinElmer Life Sciences) and detected by autoradiography on x-ray film (Kodak).

**Immunoblot Analysis**

The cells were seeded at a density of 0.5 × 10⁶ cells/ml on 100-mm Petri dishes. When cultures achieved 90–100% confluency, the serum was withdrawn, and the cells were treated with 17β estradiol (using serum-free conditions). After specific intervals noted in the text, the cells were harvested, and the extracts were prepared as previously described (33). All of the experiments were performed at least in triplicate using appropriate vehicle controls. The cell extracts were electrophoresed on SDS-PAGE gels. Gel proteins were electrophoretically transferred to a polyvinylidene difluoride membrane (wet transfer). The blot was blocked with 5% nonfat dry milk or 1% bovine serum albumin at room temperature for 1 h. The blots were incubated at 4 °C overnight with primary antibodies to ERK-1, phosphotyrosine, ERα, tuberin (C-20), actin, (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), PDGFRβ (R & D Systems), phospho-S6, S6, phospho-ERK-1/2 (Cell Signaling Technology); washed, and incubated for 1 h with appropriate secondary horseradish peroxidase-conjugated antibody (Cell Signaling Technology). The chemiluminescent signal was detected using ECL reagents (Cell Signaling Technology) and developed using autoradiography. Denitometric semiquantitative analysis was performed by ImageQuant software (Molecular Dynamics, Sunnyvale, CA). The data were expressed as fold activation relative to control.

**Statistical Analysis**

The results are expressed as the means ± S.D. Statistical analysis using a nonparametric test (Mann Whitney U) was performed using SPSS software. The significance was assumed at a p value of <0.05.

**RESULTS**

**Tuberin and ERα Form an in Vivo and in Vitro Complex**

To investigate the regulatory mechanisms involved in the cellular response to estrogen, we investigated a potential interaction between tuberin and ER. To demonstrate in vivo complex formation between ERα and tuberin, mammalian constructs for GFP-TSC2, GFP-TSC2-C672 (C-terminal 672 amino acids of TSC2), or ERα were expressed in HEK293 cells by adeno-annorally mediated transfer. Cell lysates were immunoprecipitated with GFP antibody and subjected to immunoblot analysis for ERα. Fig. 1A demonstrates significant complex formation between tuberin and ERα. Furthermore, complex formation is also observed in cells expressing ERα and TSC2-C672, suggesting that binding of ERα occurs within the C-terminal 672-amino acid portion of the tuberin molecule. In a series of experiments, mammalian constructs for full-length TSC2 or ERα were expressed in HEK 293 cells. The cells were treated with E2 (10 nM) (18h-24h). The cell lysates were immunoprecipitated with anti-ERα and analyzed by immunoblotting for tuberin. Complex formation between TSC2 and ERα was again observed independent of E2 treatment (Fig. 1B). In vitro complex formation between ERα and tuberin was next inves-
tigated. Glutathione-Sepharose-bound GST-TSC2-C672 was incubated with baculovirus-expressed human recombinant ERα or ERβ in the presence and absence of E2 (10 nM). Glutathione-Sepharose-bound proteins were subjected to immunoblot analysis for ERα or ERβ. Complex formation between TSC2-C672 and ERα was observed. E2 appeared to reduce complex formation somewhat, but complex formation was observed in the absence of ligand, suggesting that ligand was not required for this interaction (Fig. 1C). In contrast, no significant complex formation between TSC2-C672 and ERβ was demonstrated. These results indicate that tuberin and ERα are capable of forming a complex in vitro and in vivo at high expression levels and that the interaction occurs between the C-terminal residues of tuberin and ERα. Furthermore, binding of tuberin to the estrogen receptor is ERα-specific and is not E2-dependent.

**Tuberin Binds to ERα in Vivo and Modulates Growth of ELT-3 Cells**

ELT-3 cells are tuberin-null smooth muscle cells that constitutively express ERα. We used this cell line to examine the potential in vivo interaction between tuberin and ERα in detail.

**Tuberin Binds to ERα in ELT-3 Cells**—Full-length TSC2 was expressed in ELT-3 cells using retrovirologically mediated transfer of the TSC2 gene (TSC2-ELT-3 cells). Tuberin expression in stable cell lines was assessed in TSC2 ELT-3 cells by subjecting cell lysates to Western immunoblot with anti-tuberin antibody and compared with ELT-3 cells (ELT-3) and to ELT-3 cells expressing empty vector only (EV ELT-3 cells) (Fig. 2A). Successful expression of tuberin was observed in TSC2 ELT-3 cells, and the absence of tuberin expression was noted in ELT-3 cells and in EV ELT-3 cells. Tuberin expression in TSC2 ELT-3 cells was 3-fold (mean ± S.D.; 3.02 ± 0.46) higher than that seen in primary pulmonary fibroblasts, vascular smooth muscle cells, or vascular endothelial cells (results not shown). In vivo complex formation between tuberin and ERα was next examined in TSC2 ELT-3 cells by subjecting immunoprecipitates of ERα to immunoblot analysis for tuberin (Fig. 2B, upper panel) and immunoprecipitates of tuberin to immunoblot analysis for ERα (Fig. 2B, lower panel). Co-immunoprecipitation of ERα and tuberin was observed in TSC2 ELT-3 cells compared with ELT-3 cells and EV ELT-3 cells. Overall, these results are similar to that observed in the overexpression studies showing that ERα, under conditions of constitutive expression, binds in vivo to full-length tuberin when expressed in ELT-3 cells.

**Tuberin Expression Inhibits Estrogen-induced Growth of ELT-3 Cells**—We and others have previously demonstrated that tuberin-null ELT-3 cells grow in response to estrogen (33, 41). In contrast, growth inhibition was observed in response to estrogen in vascular smooth muscle cells that normally express tuberin (33, 42–44). Thus, we examined the effect of tuberin expression on the growth of ELT-3 cells in response to estrogen. Significant inhibition of both basal and estrogen-induced growth of TSC2 ELT-3 cells was observed when compared with ELT-3 and EV ELT-3 cells as assessed by Coulter counting (Fig. 3A) and [3H]thymidine incorporation (Fig. 4B). We also noted that in addition to the loss of a positive growth response to estrogen, TSC2 ELT-3 cells grew at a markedly slower rate than ELT-3 and EV ELT-3 cells. However, TSC2 ELT-3 cells did grow as evidenced by the incorporation of [3H]thymidine in the absence of a growth stimulus (Fig. 3B, lower panel) and in response to PDGFBB with a 2-fold increase in [3H]thymidine incorporation similar to that observed in ELT-3 and EV ELT-3 cells (Fig. 3B, upper panel), thus indicating that tuberin expression did not induce growth arrest in ELT-3 cells. Lastly, the growth effect of estrogen in ELT-3 and EV ELT-3 cells was inhibited by the ER inhibitor ICI 182,780 and not by the ERβ inhibitor THC (Fig. 3C). Neither inhibitor had any effect on the growth of TSC2 ELT-3 cells. Collectively, these data indicate that the absence of tuberin expression in ELT-3 cells is associated with estrogen-induced growth that is ERα-specific and that expression of tuberin in ELT-3 cells results in the loss of a growth-stimulating effect of estrogen.

**Tuberin Regulates Estrogen-induced Expression and Activation of PDGFRβ**

In vascular smooth muscle cells, estrogen down-regulates PDGF/PDGFR and abrogates PDGF-induced cellular growth (6, 33). In contrast, we have previously shown that estrogen-induced growth of ELT-3 cells is mediated by the autocrine activation of PDGFRβ by PDGF-BB (33). We compared the ability of estrogen to induce and activate PDGFRβ in tuberin-null and tuberin-expressing ELT-3 cells (Fig. 4A). As expected, estrogen significantly induced PDGFRβ expression in ELT-3...
and EV ELT-3 cells. In contrast, the re-expression of tuberin in TSC2 ELT-3 cells resulted in the loss of a significant PDGFRβ phosphorylation in response to estrogen. In addition, the induction of PDGFRβ by estrogen was inhibited by both the ER inhibitor ICI 182,780 and actinomycin D, suggesting that estrogen-induced induction of PDGFRβ was ER-specific and under transcriptional control (Fig. 4B). We next compared the phosphorylation of PDGFRβ in response to estrogen in ELT-3, EV ELT-3, and TSC2 ELT-3 cells (Fig. 4C). As expected, estrogen resulted in significant phosphorylation of PDGFRβ in ELT-3 and EV ELT-3 cells. In contrast, PDGFRβ phosphorylation was completely absent in TSC2 ELT-3 cells. Immunoblotting for PDGFRβ did reveal that adequate expression of PDGFRβ was precipitated in tuberin-expressing samples, indicating that the absence of PDGFRβ phosphorylation in TSC2 ELT-3 cells was not due to inadequate immunoprecipitation or inadequate expression of PDGFRβ but rather due to a true effect of estrogen. These data indicate that the re-expression of tuberin in ELT-3 cells significantly inhibits estrogen-induced expression levels and activation of PDGFRβ. Lastly, to ensure that the expression of tuberin in ELT-3 cells was not down-regulating the total PDGFRβ, we compared PDGFRβ expression in tuberin-null and tuberin-expressing ELT-3 cells. Interestingly, we found that the re-expression of tuberin in ELT-3 cells resulted in a 1.8-fold (mean ± S.D.; 1.8-fold ± 0.01-fold) increase in the expression of total PDGFRβ, an observation reported by some of us recently in murine fibroblasts (Fig. 4D) (45).

**Tuberin Expression Inhibits Activation of S6, ERK, and Estrogen-induced Activation of ERK-1/2 MAP Kinase**

It has been shown that tuberin is a key regulator of Akt-mTOR-S6K signaling (46–48). Fig. 5A demonstrates that, similar to previous observations, tuberin expression in ELT-3 cells significantly inhibits the phosphorylation of S6 (mean ± S.D.; 7.6-fold ± 1.2-fold) (47). Estrogen is known to activate ERK MAP kinase, and estrogen-induced growth of tuberin-null and tuberin-expressing ELT-3 cells. Interestingly, we found that the re-expression of tuberin in ELT-3 cells resulted in a 5.4-fold (mean ± S.D.; 5.4-fold ± 0.47) and late time points (mean ± S.D.; 5.45 ± 0.57). In contrast, ERK MAP kinase was not activated by estrogen in ELT-3 cells treated with estrogen in unstimulated ELT-3 cells (Fig. 5A). We have previously noted two phases of ERK MAP kinase activation in response to estrogen in ELT-3 cells, an early phase occurring at 15 min and a late phase that is sustained at 24–48 h. Interestingly, the late phase of ERK MAP kinase activation was mediated via the autocrine induction of PDGF-BB by estrogen in ELT-3 cells (33). We compared the temporal patterns of ERK MAP kinase activation in response to estrogen in tuberin-null and tuberin-expressing ELT-3 cells. Fig. 5B demonstrates the expected biphasic pattern of ERK MAP kinase activation following treatment with estrogen in ELT-3 and EV ELT-3 cells. The mean activation of ERK MAP kinase in EV ELT-3 cells was 5-fold at both early (mean ± S.D.; 5.40 ± 0.47) and late time points (mean ± S.D.; 5.45 ± 0.57). In contrast, ERK MAP kinase was not activated by estrogen in ELT-3 cells treated with estrogen in the presence of rapamycin in or TSC2 ELT-3 cells. In fact, a 5-fold (mean ± S.D.; 5.0 ± 0.19) inhibition of ERK MAP kinase activation was observed in response to estrogen in TSC2 ELT-3 cells at 24 h. In parallel with our observations of the inhibitory effects of tuberin on ELT-3 cell growth and PDGFRβ activation in response to estrogen, these data indicate that the re-expression of tuberin in ELT-3 cells also significantly inhibits estrogen-induced activation of ERK MAP kinase.

**DISCUSSION**

The diverse effects of estrogen are mediated through its receptors, ERα and ERβ. The specific functions of both of these receptors are subject to tight regulation by a number of cell type-specific co-repressors and co-activators. In this study, we describe a novel function for the tumor suppressor protein tuberin, which binds to ERα and acts as a repressor of E2/ERα-
mediated cellular growth. We describe a pathway regulated by tuberin in ELT-3 smooth muscle cells where estrogen, under conditions of tuberin expression, results in a limited growth effect that is associated with inhibition of PDGFRβ and ERK activation in response to E2. In contrast, loss of function of tuberin leads to growth stimulation in response to E2 that is associated with robust activation of PDGFRβ and prolonged activation of ERK.

ER regulates ligand or non-ligand-induced gene transcription via DNA-binding domains, a property that is common to all steroid receptors (10, 49). Over the last few years, a variety of steroid receptor co-regulators have been described (11). Corepressors and co-activators bind to ER to modulate its ability to regulate gene transcription. Binding of unique co-regulators can lead to tissue-specific responses (50–52). The tuberin molecule is a large 180-kDa tumor suppressor protein with a variety of protein-interacting domains (19, 26, 28, 53–55). We have previously shown that tuberin can modulate ligand-induced steroid/nuclear receptor-mediated transcription in vitro (24–26). In this study, we now describe for the first time complex formation between the tumor suppressor protein tuberin and one of the major subtypes of estrogen receptor, ERα. Complex formation was ERα-specific with comparatively very little complex formation observed between ERβ and tuberin. However, it is possible that ERβ binds to the N terminus of tuberin or that interaction with ERβ may require additional intracellular proteins for complex formation with tuberin in vitro. Nonetheless, the specificity of tuberin for complex formation with ERα coincides with the observation that E2-mediated cellular growth of ELT-3 cells is also ERα-specific with complete inhibition of growth in response to the ER inhibitor ICI 182,780 and the lack of inhibition in response to the ERβ inhibitor, THC. In addition to these findings we also observed that ERα binds to the C-terminal end of the tuberin molecule. It has been demonstrated that the C terminus is important in regulating many functions of tuberin but in particular its tumor suppressor function (56). Together, these data demonstrate that ERα and tuberin bind in vitro and in vivo and suggest that as a tumor suppressor protein, tuberin acts as a co-repressor or inhibitor of ERα function in ELT-3 smooth muscle cells.
FIG. 5. Tuberin re-expression inhibits phospho-S6 and ERK-1/2 activation in ELT-3 cells. A, phosphorylation of S6 (IB: p-S6) and total S6 protein (IB: S6) and ERK-1/2 (IB: p-ERK) and total ERK protein (IB: ERK) were determined using Western immunoblot in ELT-3 cells (ELT-3) or ELT-3 cells expressing empty vector (EV ELT-3) and compared with tuberin-expressing ELT-3 cells (TSC2 ELT-3). Densitometric analysis is also shown. The results are expressed as fold reduction in phosphorylated S6 protein and fold inhibition of phosphorylated ERK protein, respectively, and compared with ELT-3 cells. Significance is indicated by the asterisk.

B, phosphorylation of ERK MAP kinase (IB: p-ERK) and total ERK protein (IB: ERK) was determined using Western immunoblot in ELT-3 cells (upper panel, ELT-3), ELT-3 cells expressing empty vector (upper panel, EV ELT-3) and compared with ELT-3 cells (lower panel, ELT-3) treated with estrogen in the presence of rapamycin (10 nM) and tuberin-expressing ELT-3 cells (lower panel, TSC2 ELT-3). The cells were treated with E₂ (10 nM) for the times indicated. Densitometric analysis of ERK phosphorylation by estrogen is shown. The results are expressed as fold phosphorylation of ERK compared with control levels of activation (Time 0) on triplicate immunoblots.
In this study, we observed that complex formation occurred in the absence of ligand and that the administration of 17β estradiol did not result in significant complex disruption in vivo. Given that tuberin is highly expressed in smooth muscle, this suggests that ligand-induced activation of ERα is limited by tuberin. Studies reveal that expression of tuberin is important for normal membrane translocation of specific cytosolic proteins (57). Although the localization of ERα was not examined in this study, previous data support that tuberin may exert its inhibitory effects on ERα at the transcriptional level (24–26). Thus, it could be hypothesized that, under normal conditions, tuberin limits ligand-induced localization of ERα to the nucleus for transcription. Conversely, when tuberin function is aberrant or lost, ligand-induced activation of ERα may be uninhibited, resulting in unabrogated ER-mediated gene transcription.

We also observed that when tuberin function is lost, an autocrine loop involving PDGFRβ is induced by estrogen and that this process is transcriptionally dependent. Furthermore, the kinetics of this response suggests that this effect is not an early nongenomic event but rather a later genomic event. Together with our previous observations (33), these data indicate that the loss of tuberin expression results in the emergence of an autocrine loop that is vital for estrogen-mediated growth of tuberin-null cells. Although the PDGFRβ promoter does not contain an estrogen response element (NCBI Protein Database accession number NM_0026009), it does contain a consensus binding site for transcription factors AP-1 and SP-1, which are known to directly activate transcription in response to the estrogen-ER complex (58, 59). Thus, the expression of tuberin in ELT-3 cells may mediate its tumor suppressor effect indirectly through the altered transcription of AP-1 and SP-1.

As an extension of our previous studies, we now show that the expression of tuberin in ELT-3 cells results in a cell phenotype that is not responsive to estrogen in terms of cell growth. Previous observations have shown that cellular growth from the same tissue source can respond to estrogen in a diverse fashion. For example, vascular smooth muscle cells can be stimulated or growth-inhibited by estrogen (6, 33, 42, 43, 60). Similarly, breast cancer cell growth can be estrogen-dependent or estrogen-resistant. ELT-3 cells are derived from uterine leiomyomas of the Eker rat, and our data indicate that the positive growth response to estrogen does not solely relate to the tissue source. Rather, our data show that the response to estrogen in the uterus is regulated by the expression of tuberin. In support of these findings Burroughs et al. (60) demonstrated in vivo that compared with tuberin-null leiomyomas, the growth of the normal surrounding tuberin-expressing myometrium was not dependent on estrogen. Moreover, they also noted that in vivo leiomyoma growth was due to both an increased proliferative and reduced anti-apoptotic response to estradiol. Together, these data suggest that tuberin plays a pivotal role in regulating estrogen-induced proliferative and anti-apoptotic signals to modulate cellular growth in the uterus. In fact, given the diverse growth effects of estrogen in vascular tissue, it could also be postulated that tuberin functions as a pivotal switch to regulate steroid-mediated cell growth in many estrogen-responsive tissues.

Recent studies now clearly demonstrate that tuberin is a signaling intermediate that binds to and is phosphorylated by Akt to effect downstream intermediates, mTOR and S6 (reviewed in Ref. 29). Similar to that observed by other groups, we noted that the loss of tuberin expression induces marked activation of phospho-S6. However, in this study we also show that loss of tuberin function results in significant phosphorylation of ERK MAP kinase, which is enhanced and temporally biphasic in response to estrogen. Interestingly, the early phase of ERK MAP kinase phosphorylation by estrogen was equivalently inhibited by both rapamycin and tuberin expression. This suggests that the effect of tuberin expression on the early nongenomic activation of ERK MAP kinase by estrogen lies downstream of mTOR. We also observed that the late phase of estrogen-induced ERK MAP kinase phosphorylation was also inhibited by rapamycin. This was in contrast to the continued down-regulation of estrogen-induced ERK MAP kinase activation by the expression of tuberin. Although the latter observation may be due to the overexpression of tuberin, it could be speculated that tuberin is required to inhibit downstream ligand-induced ER-mediated transcription of PDGFR/PDGF-BB with consequent limited activation of ERK. This hypothesis is supported by our previous observations that the late phase of ERK MAP kinase activation in tuberin-null ELT-3 cells was mediated by the autocrine induction of PDGF-BB (33). Tee et al. (32) have recently demonstrated that tuberin is phosphorylated by ERK MAP kinase and that inhibition of phospho-S6 by TSC2 was partially mediated by protein kinase C-dependent MAP kinase activation. Overall, our observations now show that ERK MAP kinase phosphorylation is inhibited when tuberin is expressed and associated with a poorly proliferative phenotype. In contrast, ERK protein is activated when tuberin function is lost, leading to a more proliferative phenotype and a phenotype that is also more responsive to estrogen. Although the precise relationship between MAP kinase and tuberin remains unclear, the data in our study support an additional role for tuberin in antagonizing not only the Akt-mTor-S6 pathway but also the ERK/MAP kinase pathway.

In summary, we have shown that the tumor suppressor protein tuberin binds to ERs and regulates estrogen-induced cell growth. Our data support the hypothesis that tuberin acts as a pivotal signal in estrogen-mediated cell growth and that modulation of receptor activation and intracellular signaling intermediates by tuberin plays a role in determining the growth response to estrogen. In addition, our data now add to the growing body of literature that supports tuberin as a vital signaling molecule capable of regulating multiple pathways involved in cell growth. This study provides valuable insight into the mechanisms that regulate cell growth in states of tuberin dysfunction such as tuberous sclerosis and lymphangioleiomyomatosis. Furthermore data from this study support a potential role for tuberin in regulating estrogen-mediated cell growth. Understanding altered growth regulation of ELT-3 cells could lead to novel therapeutic approaches to the treatment of not only TSC and lymphangioleiomyomatosis but also of diseases characterized by estrogen dependence such as benign and malignant tumors of the reproductive tract.

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