The FASEB Journal express article 10.1096/fj.04-3142fje. Published online April 25, 2005.

Cross-talk between tuberin, calmodulin, and estrogen signaling pathways

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ABSTRACT

Lymphangioleiomyomatosis (LAM) is a rare disease that occurs primarily in women and has been linked to both estrogen-mediated signaling events and mutations associated with the tuberous sclerosis complex 2 gene product tuberin. These two observations fostered the hypothesis that tuberin's impact on estrogen-mediated signaling might be through a direct interaction with the intracellular receptor for estrogen, estrogen receptor α (ERα). In the study presented here, tuberin was shown to co-immunoprecipitate and directly bind ERα through a domain localized within the carboxyl 73 amino acids of tuberin. This domain had previously been shown to serve as a binding domain for the intracellular calcium signaling molecule calmodulin (CaM). Competition binding studies identified a potential competitive relationship for binding of tuberin by ERα and CaM. Additionally, tuberin-ERα interactions were found to be modulated by the presence of tuberin's predominant intracellular binding partner hamartin, suggesting that tuberin-hamartin interactions negatively impact the ability of tuberin to modulate ERα-mediated gene transcription events. Cumulatively, data presented here support the hypothesis that interactions between tuberin, ERα, and CaM may play a critical role in the pathology of LAM disease.

Key words: lymphangioleiomyomatosis • tuberous sclerosis • hamartin • estrogen receptor alpha

Lymphangioleiomyomatosis (LAM), first reported over 50 years ago, is a rare disease occurring primarily in women of childbearing age. The disease manifests itself as an aberration of proliferation, differentiation, and migration of immature smooth muscle cells [reviewed in (1)]. This aberration subsequently leads to the development of numerous airflow abnormalities that promote the progression of the disease. Unfortunately, patients with the disease have a poor prognosis with an average survival of less than 10 years from detection of the disease.

The pathogenesis of LAM correlates with both steroid hormone fluctuations and the set of genetic diseases collectively termed the tuberous sclerosis complex (TSC). The fact that LAM is

found almost exclusively in post-pubescent but pre-menopausal women suggests that it may coincide with estrogen hormone signaling pathways. This theory is further supported by the observations that pregnancy in women with LAM seems to exacerbate the disease, while anti-estrogen therapies, including oophorectomy, ovarian irradiation, and progesterone administration, often lessen the severity of the disease (1).

TSC is an autosomal dominant neurocutaneous disease that leads to the development of hamartomatous tumors in a variety of tissue types (2). TSC has been genetically mapped in humans to two distinct loci: TSC1 and TSC2 (3). The TSC1 gene encodes hamartin, a 130 kDa protein containing 1164 amino acids, whereas the TSC2 gene encodes a 198 kDa protein called tuberin containing 1807 amino acids. These proteins have been shown to exist in a complex that is involved in regulating cell growth mediated by the mTOR/S6K and β-catenin signaling pathways [reviewed in (4)]. Tuberin and hamartin also appear to maintain distinct cellular responsibilities, with evidence for hamartin regulation of cell adhesion events through its association with Ezrin/Radixin/Moesin proteins and the GTPase Rho (5), and for tuberin involvement in steroid mediated transcription events (6).

Steroid hormones, including estrogens, mediate their activities through the binding and activation of an intracellular receptor. Estrogen receptor α (ERα) belongs to a superfamily of ligand-inducible transcription factors that, upon binding ligand, undergo a conformational change that facilitates their association with both DNA and a variety of co-regulatory components [reviewed in (7)]. Along with the ability to regulate gene transcriptional activity, the unliganded and liganded states of ERα also participate in complexes of proteins that serve a variety of cellular functions. Although many of the binding partners for the estrogen receptor remain unknown, evidence suggests that this receptor modulates cell cycle progression and alters calcium responses (8).

ERα activation and tuberin signaling events also appear to be impacted by calmodulin (CaM). Both ERα and tuberin contain a predicted basic amphipathic α helix that can bind CaM (9, 10), and the interaction of CaM with ERα and tuberin has been demonstrated to have functional consequences. The calcium-dependent interaction of CaM with ERα stimulates ERα-DNA binding (11) and also confers stability to ERα by preventing its proteolysis (12). ERα-CaM interactions, therefore, serve as an intracellular switch capable of regulating ERα transcriptional activation of target genes. Similarly, tuberin has been shown to modulate steroid receptor-mediated transcriptional events (6), and deletion of the CaM binding domain of tuberin abolishes the ability of tuberin to modulate this transcriptional activity. These studies establish a functional role for tuberin-CaM interactions and collectively identify CaM as a potential link between estrogen receptor and tuberin signaling pathways.

The correlation between tuberin dysfunction and estrogen stimulation as contributing factors in the development and progression of LAM disease suggests a link between these two proteins and their involvement in cellular signaling cascades. Here, we demonstrate direct interactions between tuberin and ERα and localize these interactions to the region of tuberin's CaM binding domain. Experimental evidence is also provided for a competitive relationship between ERα and CaM for binding to tuberin and a potential mechanism for tuberin's modulation of ERα-mediated transcription events. The identification of these protein-protein interactions provides

novel insights into how aberrations in tuberin function may directly correlate with TSC and LAM disease pathologies.

MATERIALS AND METHODS

Reagents and materials

General chemicals, unless otherwise noted, were purchased from Sigma (St. Louis, MO). Protease inhibitor cocktails were purchased from Roche Diagnostics (Indianapolis, IN). Restriction enzymes and other DNA-modifying enzymes were purchased from New England Biolabs (Beverly, MA). Recombinant human ERα was purchased from PanVera (Madison, WI). GSTrap FF columns used for the purification of GST tagged proteins were purchased from Amersham Biosciences. Antibodies used for the detection of tuberin, ERα, and GFP were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA); those used to detect CaM, from Upstate Biotechnology, Inc.; those used to detect the FLAG epitope, from Sigma; and those used to detect hamartin, from Zymed Laboratories, Inc. (South San Francisco, CA). Secondary antibodies were purchased from both Cell Signaling Technology, Inc. (Beverly, MA) and Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA), and the antibody for the detection of thiolase was a generous gift from Sidney Whiteheart (University of Kentucky). CaM was purified from bovine testes as described previously (13). CaM-Sepharose was prepared by using the purified bovine testis protein exactly as described (14).

Plasmid constructs

The GST fusion constructs and the adenovirus TSC2 constructs were described previously (6, 10, 15). Mammalian expression constructs for pRSV-hERα, pED-TSC2 (10), and pRSV-β-galactosidase (pRSV-β-Gal) are described elsewhere, as is the pBL-ERE-tkLuc luciferase reporter construct (16). The pRSV-TSC1 and pEGFP-TSC1 mammalian expression constructs were created by subcloning a *Kpn* I fragment from a pBSK-TSC1 clone (a generous gift from David Kwiatkowski, Brigham and Women's Hospital), containing the full-length TSC1 coding sequence, into the *Kpn* I sites of the previously described pRSV (16) and pEGFP-3 (Clontech, Inc.) mammalian expression vectors.

Recombinant protein purification

Glutathione-S-transferase (GST) and GST-TSC2 fusion proteins were prepared from BL21-RP *Escherichia coli* cells as described previously (17). Briefly, transformed cells were grown to log phase and protein expression was induced by the addition of IPTG. Cells were lysed by repeated freeze-thaw followed by disruption in a French Press. Lysates were clarified by ultracentrifugation at 100,000 × *g* for 1 h and GST-tagged fusion proteins were purified by fast-performance liquid chromatography on a GSTrap FF column. Eluates were dialyzed and the dialyzed proteins were aliquoted and stored at –70°C until use. Protein concentrations were determined by BCA analysis as per the manufacturer's instructions (Pierce Biotechnology, Inc.).

Immunoprecipitation assays

LAM and HASM (human airway smooth muscle) cells were cultured as described previously (18). For co-immunoprecipitation of endogenous ERα and tuberin from cell lines, the cells were

harvested in IP lysis buffer (50 mM Tris-HCl, pH 7.5; 150 mM NaCl; 5 mM EDTA; 1% NP-40; 0.1% SDS; 1× protease inhibitors). For co-immunoprecipitation using rat brain tissue, rat brains were homogenized in homogenization buffer (50 mM HEPES pH 7.4; 10% glycerol; 2 mM EDTA; 2 mM EGTA; 1 mM DTT; 1× protease inhibitors) using a Dounce homogenizer. Homogenates were centrifuged at 100,000 × *g* for 1 h. For co-immunoprecipitations, 5 mg of centrifuged extract was brought up to a total volume of 500 µl in 1× IP lysis buffer. Homogenates were pre-cleared (30 min at 4°C) of Sepharose binding proteins and subsequently incubated (overnight at 4°C) with ERα antibody or rabbit polyclonal non-specific IgG antibody (5 µg antibody/5 mg of extract). IgG complexes were purified by incubation (2 h at 4°C) of extracts with 25 µl of protein G Sepharose beads (Amersham). Bead-retained protein complexes were washed 10× with 10 volumes of IP lysis buffer and eluted with 2×-SDS electrophoresis buffer. The released proteins were analyzed by Western blot probing with antisera specific for tuberin and ERα.

For immunoprecipitation assays using HEK293 cells, these cells were first either transfected with mammalian expression constructs for Flag-TSC2, hamartin, and/or ERα using a standard calcium phosphate delivery system (Fig. 5*A*), or infected using recombinant adenovirus expressing GFP-TSC2-C672, ERα, or TSC1 (Fig. 5*B*). Lysates were generated from transfected and infected cells using IP lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 0.1% SDS and 1× protease inhibitor). Protein concentrations were determined by BCA analysis. The immunoprecipitations were performed by adding either 50 µl of a 50% slurry of Flag-Agarose (Sigma, Inc.) or GFP-Agarose (Vector Laboratories, Inc., Burlingame, CA) to 1 mg of total lysate from above, and incubating for 1 h at 4°C. The bead-retained protein complexes were washed 5 times in IP lysis buffer, eluted, separated by SDS-PAGE, and transferred to nitrocellulose membranes. Western blot analyses were performed probing with antibodies for hamartin and ERα. Additionally, 50 µg of total cell lysate from each transfection or infection was also analyzed by Western blot to monitor for total protein levels of tuberin, hamartin, ERα and thiolase as indicated.

Protein pull-down assays

Pull-down assays were performed as described previously (17). Briefly, Sepharose-bound GST-tagged proteins, Sepharose-bound CaM, or Sepharose-bound Flag antibody, suspended in affinity precipitation buffer [APB: 50 mM Tris-HCl pH 7.5; 150 mM NaCl; 10% glycerol (v/v); 1% Nonidet-40 (v/v)] were incubated (30 min at 4°C) with various recombinant proteins or cell extracts as described in Results and in the figure legends. The beads were recovered by centrifugation in a microfuge and washed five times with APB; the bound proteins were analyzed by Western blot analyses probing with antisera for specific pull-down products as indicated in figure legends.

For pull-down analyses used in competition studies, Sepharose-bound target proteins (e.g., GST-tuberin or CaM) were preloaded with a binding partner by incubation of Sepharose-bound proteins with a molar excess of binding partner. Bead-retained proteins were washed 5 times with APB and incubated overnight at 4°C with molar excesses (as defined in the Results section) of competitor protein. Beads were processed as described above and analyzed by Western blot probing for either the presence of target protein, binding partner and/or competitor.

Gel mobility shift assays

GMSAs were performed essentially as described previously (17). Briefly, a double-stranded oligonucleotide encoding an estrogen response element (ERE) (5′–TCAGGTCACAGTGACCT–3′) and containing a 5′ (GATC) overhang was radioactively labeled with [α-^{32}P]-dCTP using a standard Klenow fill-in reaction (19). Labeled response element was incubated with 25 μg of HepG2 nuclear extract, recombinant ERα (1μg), recombinant tuberin (10 μg), and/or CaM (1 μg) in the presence of 10 nM 17-β-estradiol (E_2) as indicated. The response element-protein complexes were separated by electrophoresis on non-denaturing 5% polyacrylamide gels and visualized by autoradiography.

Mammalian transcription assays

In vitro transcription assays using a standard calcium phosphate precipitation protocol were performed as described previously (6). Briefly, 90% confluent 10 cm plates of HEK cells were co-transfected with 5 μg of pBL-ERE-tkLuc luciferase reporter, 5 μg of a pRSV-β-Gal normalizing plasmid, 1 μg of pRSV-hERα, 1 μg of a pFLAG-TSC2 plasmid, and/or 1 μg of a pEGFP-TSC1 plasmid. The total amount of plasmid per 10 cm plate transient transfection was brought to 20 μg, equilibrating all plasmids by the addition of appropriate amounts of pRSV, pEGFP, pFLAG, and pUC plasmids. Transfections were incubated with DNA-calcium phosphate precipitates for 6 h and washed with PBS, and cells were replated (5×10^5 cells/well) into six wells/10 cm plate of a 96-well plate. The remainder of the cells were replated into a 10 cm plate. Cells were incubated in the presence of 10 nM 17-β-estradiol (E_2) for 24 h. After 24 h, induced luciferase and normalizing β-Gal activities were determined as described previously (6). Activity was expressed as the average of sextuplet luciferase activities normalized to the average β-Gal rate [average luciferase response/(average β-Gal response/minute)] for these corresponding wells. Data were graphed as the standard error of the mean for the six wells analyzed per transfection.

Media was removed from the transfected cells in the 10 cm plates, the plates were washed with PBS, and the cells were lysed with the addition of 300 μl of IP Lysis buffer. Lysates were centrifuged, and protein concentrations were determined for the supernatants by BCA analysis. From each transfection 50 μg of total protein extract or lysates were separated by SDS-PAGE and analyzed via Western blots probing for the presence of ERα, tuberin (anti-FLAG) hamartin (anti-GFP), and cytoplasmic thiolase.

Western blot analysis

Western blot analyses were performed as described previously (6). Briefly, proteins separated by SDS-PAGE were electroblotted onto nitrocellulose membranes (Stratagene, Inc., La Jolla, CA), blocked in TBST buffer (20 mM Tris, pH 7.5; 137 mM NaCl; 0.2% Tween-20) supplemented with 5% nonfat dry milk and incubated overnight with primary antibody diluted into TBST containing 2% nonfat dry milk. Blots were subsequently washed in TBST, then incubated with an appropriate secondary antibody coupled to horseradish peroxidase, reacted with ECL reagents as described by the manufacturers (Perkin-Elmer, Inc., Foster City, CA) and detected on X-ray film by autoradiography.

RESULTS

Identification of ERα as a binding partner of tuberin

Two prominent hallmarks of the proliferating lung smooth muscle cells found in LAM lesions are the uncharacteristic expression of ERα and the loss of expression of a functional tuberin (1). Previous studies from our laboratory identified a novel interaction between ERα and tuberin (15). To investigate the potential physiological relevance of these in vitro studies, co-immunoprecipitation analyses of endogenous tuberin and ERα proteins were performed using HASM (human airway smooth muscle) cells, LAM cells (Fig. 1*A*) and whole rat brain homogenates (Fig. 1*B*). HASM cells, representing normal human lung smooth muscle cells, lack ERα expression (Fig. 1*C*). Conversely, the LAM cell line used in this study expresses detectable amounts of ERα (Fig. 1*C*), and a tuberin protein containing a 45 amino acid in-frame deletion in its N terminus at a region previously shown to be required for hamartin binding (18) (Fig. 1*C*). It should be noted that due to its large size (~195 kDa) and relatively small deletion, migration differences in LAM cell tuberin are indistinguishable in this system from that of the normal tuberin found in HEK293 cells (Fig. 1*C*, LAM panel and positive antibody control). To assess endogenous ERα-tuberin associations, HASM and LAM cell lysates were incubated with an ERα-specific antibody (Fig. 1*A*). Antibody-ERα complexes were purified using Protein G Sepharose beads and analyzed for the presence of tuberin and/or ERα in a Western blot analysis. As seen, the tuberin in LAM cells (Fig. 1*A*, lane 1 LAM panel) as opposed to HASM cells (Fig. 1*A*, lane 1 HASM panel), selectively co-immunoprecipitated with ERα. Based on the controls included in this study, it is obvious that a substantial component of endogenous ERα and tuberin in the LAM cell line associate with each other.

An additional experiment was performed to further investigate the tuberin-ERα endogenous relationship using whole rat brain extracts. Rat brains express detectable levels of both ERα and tuberin (Fig. 1*C*), and as seen here, the antibody to ERα also co-immunoprecipitated tuberin from rat brain extracts (Fig. 1*C*, lane 1 Brain panel). Collectively, these data demonstrate that endogenously expressed ERα and tuberin associate in cells and tissues.

ERα binding localizes to the carboxyl terminus of tuberin

To further investigate tuberin-ERα interactions, in vitro pull down analyses were used to assess ERα's ability to bind various domains of tuberin's carboxyl terminus.

A variety of bacterial expression constructs were developed (Fig. 2*A*) and used to generate recombinant GST-tagged tuberin fusion proteins. Recombinant proteins were bound to glutathione-Sepharose beads and assessed for their ability to bind purified recombinant ERα (Fig. 2*B*). ERα bound efficiently to the carboxyl 672 amino acid portion of tuberin (Fig. 2*B*, lane 1), but failed to bind to a similar protein construct lacking the carboxyl terminal 73 amino acids of tuberin (Fig. 2*B*, lane 2). Furthermore, binding studies using only this carboxyl 73 amino acid segment of tuberin (C-73) demonstrated efficient binding to ERα (Fig. 2*B*, lane 3).

Previous work from our laboratory identified a CaM binding domain to also exist within the carboxyl terminal 73 amino acid segment of tuberin (amino acids 1740–1757) (10). Furthermore, this domain has been shown to contain one of the most highly expressed LAM and TSC-

associated mutations, a six-amino acid in-frame deletion (Δ1746-1752). To assess potential overlap in tuberin binding domains for ERα and CaM, in vitro ERα-pull down analyses were performed using GST-tagged recombinant tuberin proteins containing either a complete deletion of the CaM binding domain (TSC2ΔCaM) or the six-amino acid in-frame deletion within the CaM binding domain (mCBD). As seen in Fig. 2*B*, both the TSC2ΔCaM (lane 2) and the mCBD (lane 4) proteins failed to bind ERα, suggesting tuberin's binding domain for ERα overlaps with its binding domain for CaM.

ERα binding is independent of CaM binding

The observation that ERα binding localizes to a similar domain as CaM binding prompted the investigation of ERα-CaM competition for binding to tuberin. In vitro competition assays were performed wherein bead-bound recombinant GST-TSC2-C672 (Fig. 3*A*) or GST-TSC2-C73 and GST-TSC2-mCBD (Fig. 3*B*) proteins were incubated with a molar excess of recombinant ERα. The residual ERα was removed by extensive washing, and a molar excess of CaM was added in the presence of 2.5 mM $CaCl_2$. The samples were incubated overnight at 4°C, washed, eluted, and analyzed by Western blot probing with an anti-ERα antibody (Fig. 3*A* and *B*). As observed above, ERα efficiently bound both TSC2-C672 (Fig. 3*A*, lane 1) and TSC2-C73 (Fig. 3*B*, lane 1), but binding was substantially reduced for the TSC2-mCBD construct (Fig. 3*B*, lane 3). It was also observed that the addition of excess CaM/Ca^{2+} was unable to disrupt either TSC2-C672-ERα complexes (Fig. 3*A*, lane 3) or TSC2-C73-ERα complexes (Fig. 3*B*, lane 2). Furthermore, as seen in Fig. 3*A*, lanes 2 and 4, as well as in all in vivo immunoprecipitation studies performed thus far, estradiol appears to have minimal impact on the interaction of tuberin and ERα. Finally, these data are also consistent with the hypothesis that interaction between tuberin and ERα, once the complex is formed, is not disrupted by the presence of excess CaM/Ca^{2+}.

A tuberin-CaM complex can also bind to ERα

Data presented above provide evidence for tuberin binding to both CaM and ERα at a domain that, at least in part, is overlapping. Furthermore, published data clearly demonstrate that ERα can also independently bind CaM (9). These data therefore pose two rational models for tuberin-CaM-ERα interactions. One would be a cooperative complex between tuberin, CaM, and ERα using independent binding sites on ERα, CaM, and/or tuberin. The other would be a competitive model based upon binding affinities and/or steric hindrance that involves shared or overlapping binding sites on ERα, CaM, and/or tuberin. To investigate these possibilities, a series of competition experiments were performed. In an initial study, glutathione Sepharose-bound GST-TSC2-C73 was preloaded with bovine CaM, to which a molar excess of recombinant ERα was added (in the presence or absence of calcium). Unbound ERα was removed by extensive washing, and bead-retained proteins were eluted and analyzed in Western blots by probing with antibodies for detection of CaM, ERα, and tuberin (Fig. 4*A*). As seen here, both CaM and ERα were pulled down effectively in the presence of Ca^{2+} when added either alone (lanes 1 and 2) or when present together (lane 3). These data, coupled with the data in Fig. 3, suggest two reasonable hypotheses. The first is that ERα added in excess displaces CaM from GST-TSC2-C73, which then binds to ERα. Alternatively, although these domains partially overlap, it is possible that ERα binding to tuberin sterically blocks tuberin's CaM binding domain, but CaM binding to tuberin may not be able to effectively block tuberin's ERα binding domain. Both of

these scenarios are supported by the intriguing observation that ERα can bind tuberin in a calcium-independent manner (lanes 4–6), exposing a possible mechanism for discriminating CaM and ERα binding to tuberin.

Increasing the preload of ERα on tuberin decreases CaM binding

To further investigate tuberin's preference of binding for ERα and CaM, and perhaps distinguish between the two hypotheses presented above, an additional competition assay was performed. Here, GST-TSC2-C672 was prebound to glutathione Sepharose to which 0, 0.5, 1, and 2 molar equivalents of ERα were allowed to bind to form a tuberin-ERα complex. Excess or unbound ERα was removed by extensive washing followed by the addition of a fivefold molar excess of CaM in the presence or absence of calcium as indicated. Unbound CaM was removed by extensive washing and bead-retained proteins were analyzed by Western blots for the presence of tuberin, ERα and CaM (Fig. 4*B*). As seen here, increasing the preload of ERα on tuberin proportionately decreases the ability for CaM binding. It is important to note that this competition assay varies from Fig. 4*A*, in that tuberin is preloaded with ERα as opposed to CaM. The loss of CaM in these complexes and the proportionate increase in ERα binding would support the hypothesis that ERα binding to tuberin sterically hinders CaM binding by effectively masking the CaM binding domain on tuberin. Collectively, these data best support a competitive model in which ERα can prevent CaM binding, but only when ERα binds to tuberin first. The data also suggest that when CaM is already in association with tuberin, ERα's impact on CaM binding is diminished simply because CaM may remain bound to tuberin or alternatively may be displaced from tuberin and rebind ERα as mentioned above (Fig. 4*A*).

Tuberin ineffectively competes with ERα for binding to CaM

Like tuberin, ERα has been reported to contain an amphipathic helical CaM binding domain (9), and this domain appears to be critical for ERα-mediated gene expression events (11). Therefore, it was important to determine whether tuberin could displace ERα bound to CaM. Accordingly, CaM-Sepharose was preloaded with a saturating amount of recombinant ERα in the presence of calcium and subsequently incubated with a molar excess of various recombinant tuberin protein constructs (Fig. 4*C*). After extensive washing, CaM-Sepharose retained proteins were eluted and analyzed by Western blot probing with an anti-ERα antibody. Neither the wild-type nor mutant constructs of the carboxyl terminal portion of tuberin could alter ERα association with CaM-Sepharose when compared with the addition of GST alone (Fig. 4*C*, lane 5). Thus, it appears that although ERα effectively competes with CaM for binding tuberin, presumably owing to their common site of interaction, the yet-to-be-defined binding site on ERα for tuberin is distinct from that for CaM binding.

Hamartin binding to tuberin effectively competes with ERα but not CaM

Tuberin and hamartin have been genetically linked to the pathogenesis of TSC and have been shown to complex in a phosphorylation-specific manner (4, 20), an event that has been demonstrated to play an important role in tuberin's ability to modulate mTOR signaling pathways (21, 22) and tuberin's ability to localize to the nucleus (23). We therefore investigated the role of tuberin-hamartin complexes in the binding of ERα and CaM (Fig. 5). Human embryonic kidney 293 (HEK293) cells were transfected with mammalian expression constructs for a Flag-tagged

tuberin (F-TSC2), hamartin (TSC1), and/or ERα. After 24 h, cells were lysed and incubated with anti-Flag antibody covalently linked to Sepharose beads. Beads were washed, and bead-bound complexes were eluted and analyzed by Western blot probing with antibodies against ERα, tuberin, and hamartin (Fig. 5*A*). Flag-tagged tuberin was observed to efficiently complex with ERα, and this complex formation was blocked completely by the overexpression of hamartin (Fig. 5*A*, lane 5). These data show that tuberin-hamartin complexes do not bind ERα.

To further validate this observation, HEK293 cells were infected with adenovirus containing expression constructs for a non-hamartin binding amino terminal deletion of tuberin (GFP-TSC2-C672), hamartin, and ERα. Eighteen hours post-infection, cells were lysed and incubated with anti-GFP agarose beads. The beads were washed as described above, and the resulting bead bound protein complexes were analyzed by Western blot analysis, again probing for tuberin, ERα, and hamartin (Fig. 5*B*). These data clearly demonstrate that hamartin overexpression fails to alter ERα binding to tuberin when the domain responsible for hamartin binding has been removed from tuberin. Of note, the hamartin blot is not included here simply because this amino terminal deletion mutant of tuberin fails to bind hamartin and, as expected, resulted in no binding. Collectively these data suggest that tuberin-hamartin interactions are capable of altering ERα binding to tuberin.

Tuberin-hamartin complexes were also assessed for their ability to associate with CaM. In vitro pull-down assays were performed by incubating whole rat brain homogenate supernatant (5 mg total protein) with CaM-Sepharose (Fig. 5*C*). CaM-Sepharose bound proteins were washed extensively, eluted, and analyzed by Western blot probing with antibodies specific for the detection of tuberin and hamartin. As seen in Fig. 5*C* (lanes 1 and 2), CaM-Sepharose pulled down both tuberin and hamartin from rat brain homogenate in a calcium-dependent manner. To date, no CaM binding domain has been reported for the hamartin protein, and a computer analysis of the hamartin amino acid sequence was unable to identify an amphipathic α-helical domain that might serve as a CaM binding domain. Therefore, in the absence of any identifiable CaM binding domain on hamartin, these data suggest that the detection of hamartin in this experiment was due to CaM's ability to bind tuberin in complex with hamartin.

Tuberin inhibits ERα-DNA interactions

ERα modulates gene expression patterns through direct binding of DNA and regulation of transcription. To evaluate the potential functional consequences of tuberin interactions with ERα, tuberin was tested for its ability to modulate ERα-DNA binding events. Nuclear extracts from HepG2 cells, supplemented with recombinant ERα, were used in gel mobility shift assays (GMSA) to evaluate the binding of ERα complexes to a radiolabeled ERE-containing oligonucleotide (Fig. 6*A*). As seen in previously published studies using this ERE sequence (11), HepG2 cell extracts contain a non-specific, faster migrating ERE binding complex (Fig. 6, ERE complex), which does not contain ERα as determined by supershift analyses. An ERα-specific DNA complex is formed upon the addition of recombinant ERα to the GMSAs (Fig. 6*A*, lane 2), and this complex is supershifted upon the addition of anti-ERα antisera (Fig. 6, lane 6). Furthermore, the formation of this complex was enhanced upon the addition of CaM/Ca^{2+} (Fig. 6*A*, compare lanes 2 and 9) in accordance with previously published data (11). Interestingly, the addition of bacterially expressed GST-TSC2-C672 to these extracts resulted in a complete loss of all complex formation (Fig. 6*A*, lanes 4, 8 and 10). Of note, GST-TSC2-C672 also efficiently

disrupted the non-specific ERE complex (Fig. 6*A*, lanes 4, 8 and 10), suggesting that tuberin may be sequestering yet unidentified components necessary for formation of the complex.

An extension of this study was performed to investigate the dose-dependent effects of tuberin-C672 on ERα-DNA complex formation (Fig. 6*B*). Reaction conditions were identical to those detailed in Fig. 6*A* except that increasing concentrations of GST-TSC2-C672 were added to the reaction. As seen here, increasing concentrations of tuberin proportionately reduced ERα-DNA complex formation.

Similar GMSAs examining ERα complex formation were performed (Fig. 6*C*) to evaluate the effects of various recombinant tuberin proteins, including a GST-TSC2-C73 protein, a GST-TSC2-C73 protein containing a six-amino acid in-frame deletion of its CaM binding domain (GST-TSC2-mCBD), and a GST-TSC2ΔCaM protein consisting of 672 amino acids at the carboxyl terminal of tuberin but lacking the last 73 amino acids that encompass the CaM/ERα binding domain. Inclusion of the GST-TSC2-C73 tuberin fragment in the GMSAs, like the GST-TSC2-C672 fragment, resulted in the complete loss of the ERα-DNA complex formation (Fig. 6*C*, lanes 5–8). However, the addition of the GST-mCBD and GST-TSC2ΔCaM fragments had very little effect on ERα-DNA binding interactions (Fig. 6*C*, lanes 9–12 and 13–16). Collectively, these data support the hypothesis that tuberin is sequestering a critical component(s) required for complex formation on an ERE containing DNA fragment and, as suggested by the binding data presented above, this is most likely through tuberin's ability to directly bind ERα and/or CaM.

Hamartin binding impacts tuberin modulation of ERα-mediated transcription

Tuberin overexpression has previously been shown to repress ERα-mediated transcription events (10), and tuberin's association with hamartin appears to play an important regulatory role with respect to its ability to modulate intracellular signaling pathways (21, 22). Furthermore, since the data in Fig. 6 demonstrate that tuberin's binding to ERα is blocked by hamartin, it would be expected that tuberin-hamartin associations would repress tuberin's influence on ERα-mediated transcription. To investigate this possibility, ERα-specific in vitro transcription assays were performed in the presence and absence of overexpressed tuberin and hamartin. Mammalian expression constructs for ERα, TSC2, and/or TSC1 genes were co-transfected into HEK cells along with an ERE-driven luciferase reporter gene and a β-galactosidase (β-Gal) normalizing plasmid. After 24 h the cells were lysed and assayed for luciferase and β-Gal activity (Fig. 7*A*). As seen here, and as previously shown (10), the overexpression of tuberin represses estrogen stimulated ERα-mediated transcription by ~29% (Fig. 7*A*, compare lanes 3 and 6), a value that might initially appear to represent a very mild repression, but increases in significance when one factors in both transfection efficiencies of multiple plasmids and the evidence that tuberin only transiently localizes to the nucleus (23). Conversely, hamartin by itself had little effect on ERα-mediated transcription (Fig. 7*A*, lane 2). However, when hamartin is overexpressed together with tuberin, estrogen activation of ERα-mediated transcription is rescued to ~110% of that seen with ERα alone (Fig. 7*A*, lane 7). Modulation of reporter gene expression appears to be a direct reflection of transcription as apposed to overexpression, as suggested by Western blot analyses of ERα, tuberin, and hamartin expression when normalized to cytoplasmic thiolase expression (Fig. 7*B*). These data, together with the above binding studies, suggest that intracellular tuberin-hamartin complexes cannot bind ERα and modulate ERα genomic signaling events.

DISCUSSION

One of several hallmarks used to characterize the proliferating smooth muscle cells of LAM lesions is the uncharacteristic expression of ERα. This observation coincides with a loss of tuberin function and may provide a possible explanation for why increases in estrogen signaling propagate the uncontrolled cell growth characteristic of LAM disease (1). In studies presented here, in vitro pull-down assays were used to demonstrate a direct interaction between tuberin and ERα. These data coincide with previously published studies that demonstrate both the in vivo and in vitro complexing of tuberin with ERα (15). Tuberin also has been shown to bind a variety of steroid/nuclear receptor family members (6), and overexpression of tuberin can be shown to modulate in vitro transcription events mediated by these receptors (6, 10). Cumulatively, these data, together with previously published studies demonstrating a potential for nuclear localization of tuberin (23), would support a direct role for tuberin in the modulation of ERα-mediated gene expression.

The carboxyl terminus of tuberin appears to play a critical role in both its regulatory functions and in disease pathology. A GTPase activating protein homology domain, two transactivation domains, and a CaM binding domain have been localized to within the carboxyl 644 amino acids of tuberin. Furthermore, this region of the TSC2 gene has recently been shown to be a hot spot for a variety of mutations associated with the development of LAM (24) and TSC (25). Of relevance to the studies reported here, Noonan et al. (10) recently presented evidence for a CaM binding domain in tuberin that localizes to a 16-amino acid amphipathic sequence within 73-amino acids of tuberin's carboxyl terminus. This domain was shown to directly bind CaM and to play an essential role in tuberin's ability to modulate transcription mediated by steroid/nuclear receptor family members (10). Studies presented here identify a binding domain for ERα that overlaps with this previously described CaM binding domain. Furthermore, mutations in this domain were shown to impact both ERα's ability to bind tuberin and ERα's ability to bind DNA. Competition studies analyzing tuberin's preference of binding for ERα and CaM suggest a model in which CaM binding to tuberin is impacted by the presence of ERα, while tuberin-ERα interactions are minimally impacted by the presence of CaM. These data would support the hypothesis that tuberin's binding coefficient for ERα is somewhat stronger than that of CaM and that either the availability and/or concentration of these binding partners may play an important role in defining their association with tuberin. Alternatively, CaM is a protein mediator of calcium signaling events, and data presented here suggest that ERα and CaM binding to tuberin can be discriminated through the presence or absence of calcium. Finally, tuberin has been shown to be a target for a variety of kinases and other binding partners (4, 26) and that it can transiently localize to the nucleus (23). Similarly, CaM (27) and ERα (7) have also been shown to associate with a variety of signaling molecules and undergo substantial conformational changes upon binding specific activating agents. The possibility that protein modifications (e.g., phosphorylation) may play a critical role in defining the accessibility of the binding domains and/or availability of the binding partners remains open and is currently under investigation. Cumulatively, these data provide the first evidence and a possible mechanism for direct crosstalk between tuberin, CaM/Ca^{2+}, and estrogen signaling pathways, and because all three of these proteins have been identified in both cytoplasmic and nuclear compartments, there exists support for their involvement in both nongenomic and genomic signaling pathways.

Previous studies have demonstrated a critical link between ERα-mediated gene expression events and CaM (12, 28). Similar to what has been published for tuberin-ERα complexes (15), ERα-CaM interactions appear to be restricted to the ERα isoform (9). Furthermore, calcium-dependent interaction of CaM with ERα has been shown to stabilize the estrogen receptor protein against degradation (12), while inhibition of CaM virtually eliminates estrogen-stimulated transcriptional activation (28). Moreover, CaM serves as a selective modulator of estrogen receptor activity by enhancing ERα's ability to bind to its respective DNA response element (11). In studies presented here, ERα complex formation at an ERE-specific DNA sequence appears to be substantially disrupted in the presence of tuberin. These data, coupled with previous studies demonstrating tuberin's ability to transiently localize to the nucleus, provide a viable mechanism by which tuberin may function to inhibit ERα-mediated transcription events through either direct binding of ERα, sequestration of nuclear CaM, or both. Cumulatively, these data suggest that tuberin, ERα, and CaM function in a common signaling capacity that ultimately regulates estrogen-mediated gene transcription.

Tuberous sclerosis and LAM have been linked to mutations occurring in the TSC2 gene. TSC2 has a complex gene structure that spans approximately 44 kb of genomic DNA comprising 41 exons and a non-coding leader exon. Currently, there are 292 reported mutations of the TSC2 gene, with the most prevalent being a 6 amino acid in-frame deletion that localizes within the CaM binding domain (E40:5238-5255del18) (29). While this mutation only represents 2% of all reported mutations, a variety of truncating mutations (nonsense, splicing, frameshift) compromise 80% of the reported TSC2 mutations, and the vast majority of these occur upstream of the CaM/ERα binding domain. In data presented here, recombinant tuberin containing the 6 amino acid in-frame CaM binding domain deletion was unable to bind ERα. Moreover, this mutation was observed to eliminate tuberin's ability to disrupt ERα complex formation in gel mobility shift assays. These data suggest that the CaM-ERα binding domain at the carboxyl terminus of tuberin may play a critical role in the pathology of TSC and LAM.

Recent studies have implicated ERα in the regulation of a variety of plasma membrane based nongenomic cell signaling pathways [reviewed in (30)] in addition to its classic ability to regulate transcription in response to estradiol. Recent studies also have shown that both tuberin and ERα can co-localize to caveolae-enriched membranes (31) and that nongenomic estrogen signaling events impact nitric oxide synthase signaling, PI(3)K/Akt activation (32), angiogenesis, and cell migration (33). Estrogen signaling has also recently been shown to regulate both the expression and activation of growth factor receptors (15), in a tuberin-dependent manner. Furthermore, nongenomic estrogen signaling also has been demonstrated to alter the phosphorylation state of tuberin, which could ultimately regulate tuberin function by impacting its association with other intracellular signaling pathways as well as its intracellular stability (34). Collectively, studies presented here, which demonstrate the direct binding of ERα by tuberin in what appears to be a physiologically relevant manner, together with the array of nongenomic signaling pathways that appear to converge on tuberin, ERα, and CaM, suggests that the interactions between these three proteins are more than adventitious and may be integral to ERα's nongenomic signaling events.

Tuberous sclerosis has also been genetically linked to mutations in the TSC1 gene. The TSC1 gene product hamartin has been shown to bind tuberin and domains specific for this binding event have been localized on the two proteins (35). Although it is believed that most cellular

tuberin and hamartin exist as a complex, cellular localization studies (23, 26) suggest these proteins also have independent functions within the cell. Data presented here suggest a central role for hamartin in the regulation of tuberin-ERα and tuberin-CaM complexes, in that hamartin blocks the binding of tuberin to ERα but not to CaM. Furthermore, hamartin was observed to rescue tuberin inhibition of ERα-mediated transcription events. These data would support the hypothesis that intracellular signaling events regulate the dissociation of tuberin-hamartin complexes and consequently downstream events involving ERα and/or CaM.

In summary, the data presented here establish a functional relationship between tuberin, ERα, hamartin, and CaM. These studies, along with published data demonstrating the functional interaction of CaM and ERα, suggest that tuberin and CaM act coordinately to regulate ERα function. These data also suggest that mutations within the TSC2 gene that lead to the disruption of tuberin-hamartin complexes or the disruption of the CaM/ERα binding domain, also disrupt tuberin's ability to regulate ERα-mediated DNA complex formation and gene transcription. The observation that in-frame deletions in this domain and/or truncating deletions upstream of the domain predominate in LAM disease would suggest a mechanism wherein tuberin's effect on differentiation and proliferation pathways in smooth muscle cells of the lung may be mediated through its ability to modulate ERα-mediated gene expression events. The collective impact that this interaction between tuberin, hamartin, CaM, and ERα may have on ERα genomic and nongenomic signaling is not entirely clear at present. However, in light of the circumstantial data supporting a functional relationship between these four proteins in both intracellular signaling pathways and disease pathogenesis, it is fair to speculate that tuberin is much more than a partner of hamartin and a regulator of mTOR/S6K signaling. Finally, although much has yet to be discovered with respect to why LAM disease occurs almost exclusively in women, the studies presented here open a new window into a viable molecular explanation for this idiosyncrasy.

ACKNOWLEDGMENTS

These studies were supported by grants from the Kentucky Lung Cancer Research Program (to D.J.N.) and grants from the National Institutes of Health HL67321 (to D.J.N.), HL071106 (to V.P.K.), and HL55301, HL64063, HL67663 (to R.A.P.). Additionally, this work was supported by grants to D.J.N and V.P.K from the LAM Foundation.

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Received September 16, 2004; accepted March 4, 2005.

Fig. 1

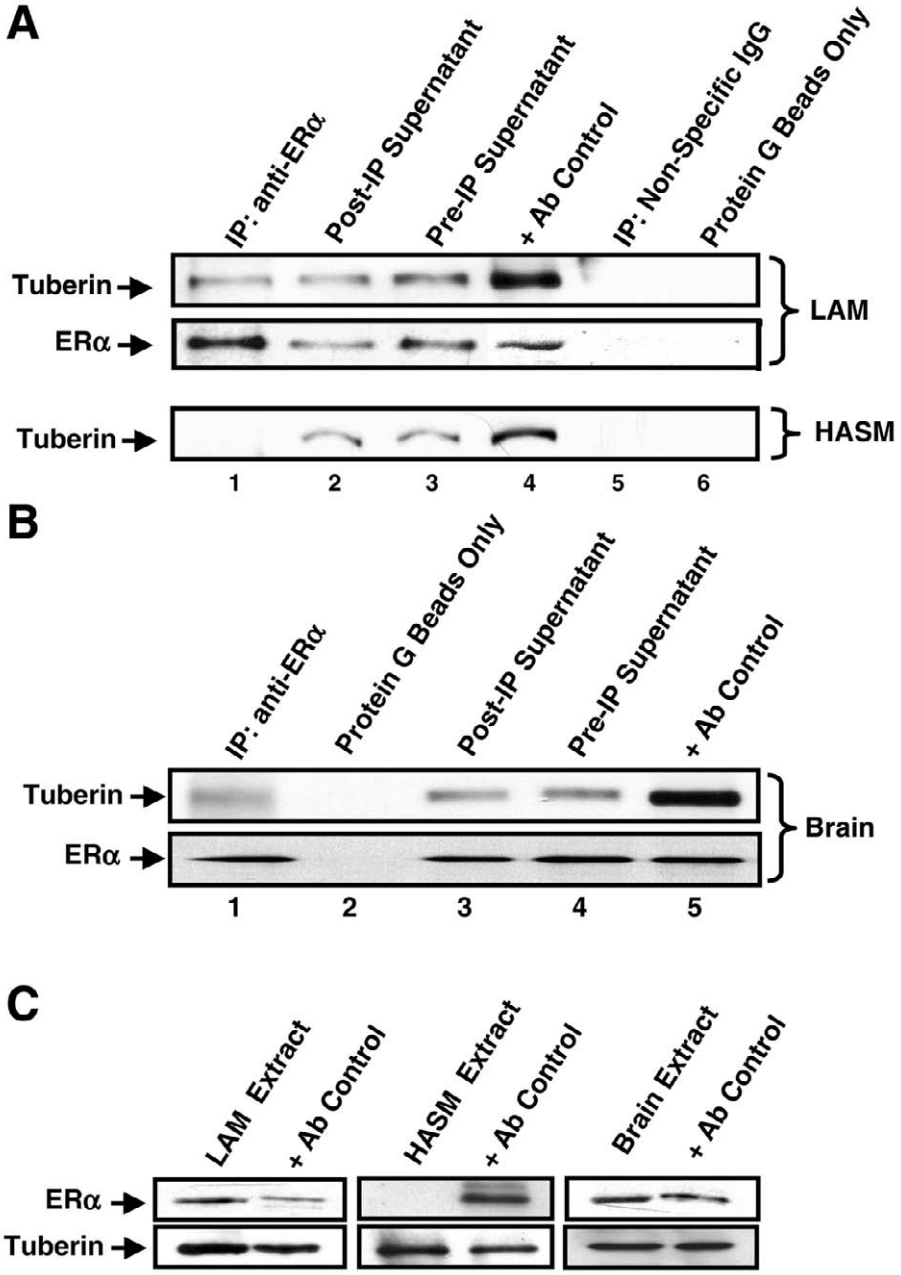

Figure 1. Identification of endogenous protein–protein interactions between tuberin and ERα. ***A***) Total cell lysates were prepared from cultured LAM and HASM cells. Either ERα antibody (5 μg) or a non-specific IgG (5 μg) was added to 5 mg of LAM or HASM total cell lysate. Resulting antibody complexes were isolated by the addition of Protein G-Sepharose beads. Bead retained proteins were solubilized in SDS-electrophoresis buffer and analyzed by Western blot, probing with an antibody specific for tuberin. The nitrocellulose membranes were subsequently stripped and reprobed for the detection of ERα (ERα panel). An aliquot of the post- and pre-IP supernatants was run to demonstrate that tuberin had been selectively removed from the total pool upon IP with the anti-ERα. Additionally, an aliquot of HEK cell lysate supplemented with 0.05 μg of recombinantly purified ERα was run as a positive antibody control for tuberin and ERα, respectively. ***B***) Whole rat brain homogenates were analyzed for co-immunoprecipitation of tuberin and ERα. ERα antibody (5 μg) was added to 5 mg of rat brain homogenate and processed as described in (***A***) above. ***C***) Aliquots of total protein extract from LAM cells, HASM cells and whole rat brain homogenate were also analyzed by Western blot for the expression levels of tuberin and ERα, respectively. All data are representative of experiments performed minimally three times.

Fig. 2

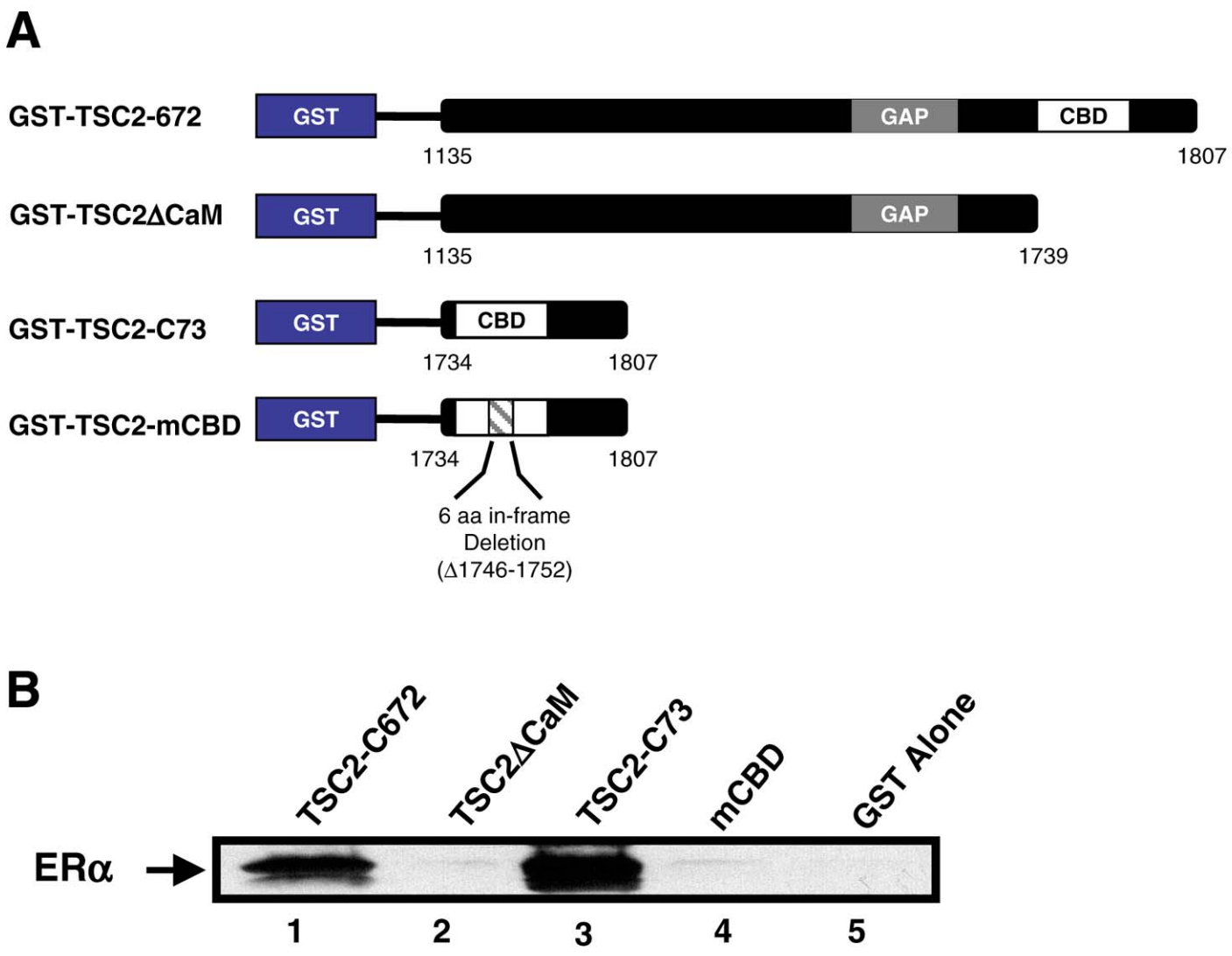

Figure 2. ERα binds the carboxyl terminus of tuberin at a domain capable of binding CaM. ***A***) A series of GST-TSC2 truncation and mutant bacterial expression constructs were used to generate recombinant tuberin proteins. ***B***) Purified recombinant GST-TSC2-C672, GST-TSC2-C73, a six-amino acid in-frame CaM binding domain deletion mutant (GST-mCBD; 10 μg of each) or a GST-TSC2ΔCaM protein consisting of 672 amino acids at the carboxyl terminus of tuberin but lacking the last 73 amino acids, were pre-bound to glutathione Sepharose. Reactions containing 30 μl of tuberin beads and 2.5 μg of recombinant human ERα were incubated at 4°C overnight, collected by centrifugation, washed, and resuspended in SDS-electrophoresis buffer to elute bound proteins. Eluates were separated by SDS-PAGE and analyzed by Western blot probing with ERα-specific antisera. Data are representative of experiments performed minimally three times.

Fig. 3

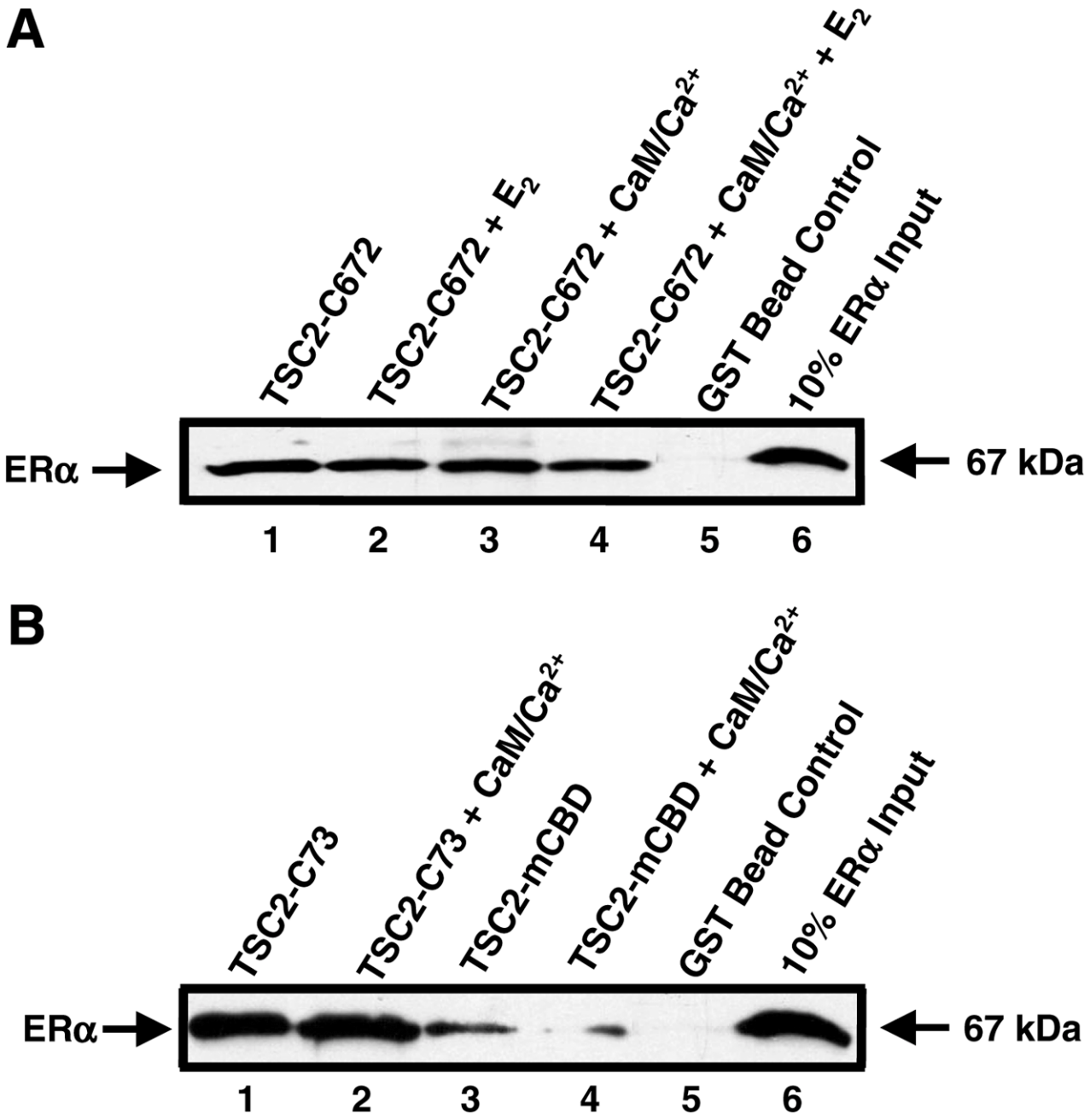

Figure 3. ERα-Tuberin interactions are not disrupted in the presence of CaM. ***A*)** Recombinant GST-TSC2-C672 (10 μg) or ***B*)** recombinant GST-TSC2-C73 and recombinant GST-TSC2-mCBD, pre-bound to glutathione Sepharose, were incubated with an excess of recombinant human ERα. Unbound ERα was removed by extensive washes, and bead-retained proteins were incubated with a vast excess (20 μg) of CaM/2.5 mM Ca^{2+} ± 17β-estradiol (10^{-8} M). Bead retained proteins were solubilized in SDS-electrophoresis buffer and analyzed by Western blots probing with an antibody specific for ERα. A lane containing 10% of the input human ERα (0.5 μg) was included as a positive control and is observed to produce an immunoreactive band of ~67 kDa (as indicated by the arrows). Data are representative of experiments performed minimally three times.

Fig. 4

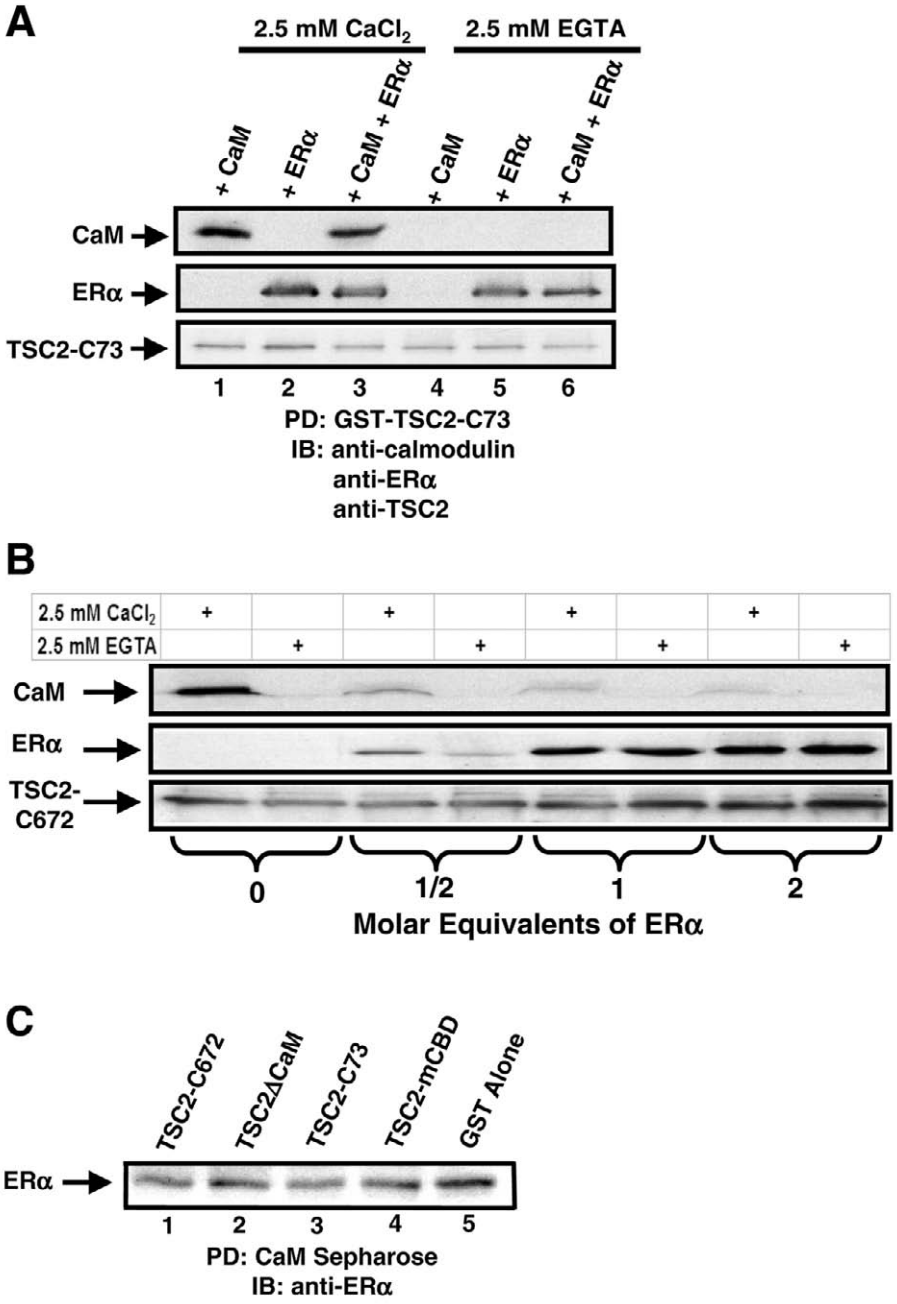

Figure 4. ERα and CaM compete for binding to tuberin. ***A*)** Bacterially expressed GST-TSC2-C73 (10 μg) bound to glutathione Sepharose beads was incubated with a 5-fold molar excess of CaM (in the presence and absence of 2.5 mM $CaCl_2$) to generate a TSC2-C73-CaM complex. The bead bound TSC2-C73-CaM complex was incubated with a molar equivalent of recombinant ERα in the presence of 2.5 mM $CaCl_2$ or 2.5 mM EGTA as indicated. Bead-retained proteins were eluted and analyzed by Western blot probing with a monoclonal anti-CaM antibody. The resulting membrane was stripped, reblocked, and reprobed with a polyclonal anti-ERα antibody as indicated. ***B*)** Bacterially expressed GST-TSC2-C672 recombinant protein (10 μg) was prebound to glutathione Sepharose followed by the addition of 0, 0.5, 1, and 2 molar equivalents of recombinantly purified ERα. Excess ERα was removed by extensive washing followed by the addition of a 5-fold molar excess of bovine CaM in the presence (2.5 mM $CaCl_2$) and absence (2.5 mM EGTA) of calcium as indicated. Bead retained proteins were collected by centrifugation and eluted by incubation with SDS-electrophoresis buffer. Samples were analyzed by Western blot probing with antibodies specific for the detection of tuberin, ERα, and CaM. ***C*)** CaM-Sepharose was preloaded with ERα protein and subsequently incubated with a molar excess of recombinant GST fusion constructs of tuberin as indicated. CaM-Sepharose retained proteins were eluted and analyzed by Western blot probing with an anti-ERα antibody. Data are representative of experiments performed minimally three times.

Fig. 5

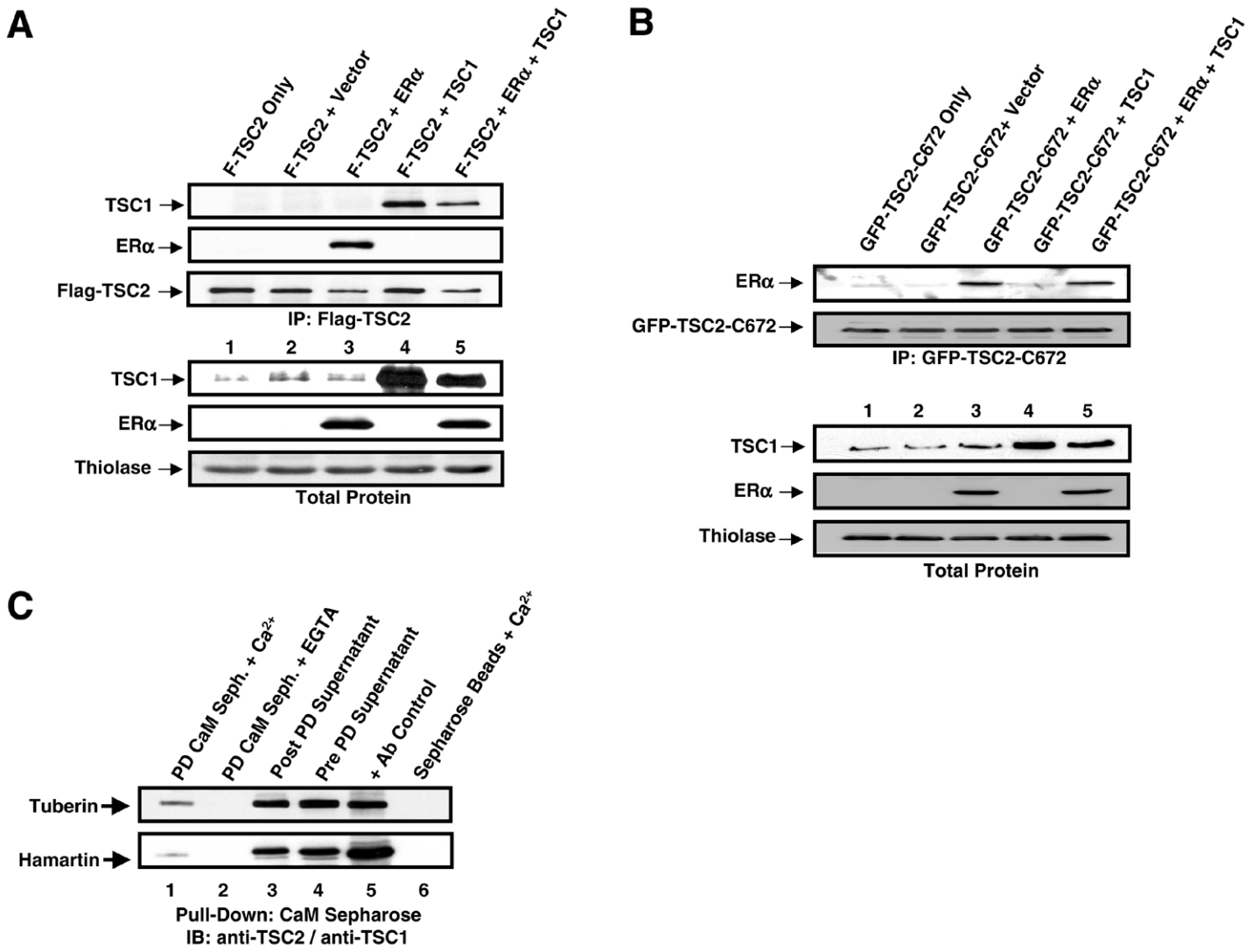

Figure 5. Hamartin inhibits tuberin-ERα complex formation. ***A*)** HEK cells were co-transfected with either a Flag tagged TSC2, human TSC1, and/or ERα mammalian expression constructs. Transfected cells were incubated for 24 h post-transfection and harvested for total cell lysate in IP lysis buffer. Total lysate (1 mg) from each transfection was incubated with Flag agarose beads. Bead-retained protein complexes were eluted, Western blotted, and probed with antibodies specific for ERα, Flag (tuberin) and hamartin as indicated. Lysate from each transfection was Western blotted and probed for total hamartin, ERα, and thiolase. ***B*)** HEK cells were co-infected with adenovirus containing expression plasmids for either a GFP tagged amino terminus deletion mutant of TSC2 (GFP-TSC2-C672), human TSC1, and/or human ERα. Infected cells were incubated for 18 h post-infection and harvested for total cell lysate in IP lysis buffer. Total lysate (1 mg) from each transfection was incubated with anti-GFP agarose beads. Bead-retained protein complexes were eluted, Western blotted, and probed with antibodies specific for ERα and tuberin as indicated. Lysate from each transfection was Western blotted and probed for total hamartin, ERα, and thiolase. ***C*)** CaM-Sepharose was utilized to pull-down CaM complexes from 5 mg of total rat brain homogenate in the presence and absence of calcium. Bead-retained proteins were eluted and Western blotted, probing with a polyclonal anti-tuberin antibody, and a monoclonal anti-hamartin antibody. Aliquots of the post (lane 3) and pre-supernatant (lane 4), as well as positive antibody controls (lane 5), were included along with a Sepharose bead only pull-down (lane 6). Data are representative of experiments performed minimally three times.

Fig. 6

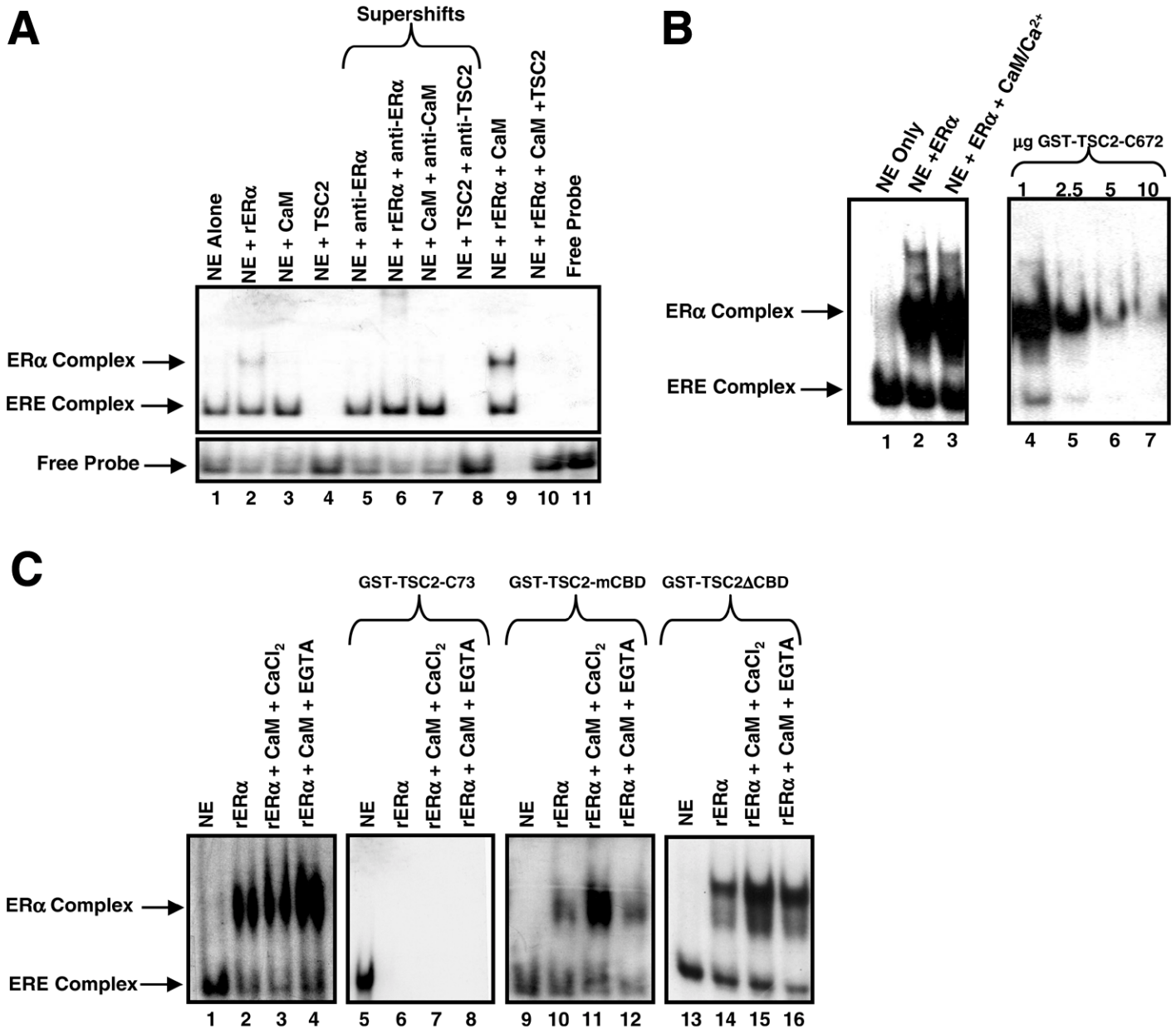

Figure 6. Tuberin inhibits ERα DNA binding events. Oligonucleotides corresponding to an ERE consensus sequence were annealed, end-labeled with ^{32}P-dCTP in a standard Klenow fill-in reaction, and used to analyze tuberin's effects on ERα binding in gel mobility shift assays (GMSA). ***A*)** The resulting labeled ERE was incubated with HepG2 nuclear extract (25 µg) and various recombinant proteins as indicated in the presence of 10 nM 17β-estradiol. DNA:protein complexes were resolved on 5% non-denaturing PAGE gels, dried, and exposed to Kodak Xar-5 film. ERE-specific complexes, ERα-specific complexes, and free probe are indicated by the arrows. ***B*)** To test the dose dependent effects of GST-TSC2-C672 on ERα-mediated complex formation, GMSAs were repeated as described in (***A***) but using constant HepG2 nuclear extract (25 µg), recombinant ERα and CaM concentrations, and increasing concentrations of GST-TSC2-C672 (1, 2.5, 5, and 10 µg, lanes 4–7). ERE- and ERα-specific complexes are indicated by arrows. ***C*)** Recombinant GST-TSC2-C73 protein (10µg), a six amino acid in-frame deletion of tuberin's CaM binding domain (GST-TSC2-mCBD), and a 672 amino acid carboxyl terminal construct of tuberin lacking the last 73 amino acids, which encompass the CaM/ERα binding domain (GST-TSC2ΔCaM)**,** were analyzed in GMSA's for their ability to alter ERα-mediated complex formation. The recombinant tuberin proteins and labeled ERE were incubated with HepG2 nuclear extract (25 µg) and in the presence or absence of recombinant ERα, CaM, calcium, or EGTA as indicated, and analyzed as above. ERE- and ERα-specific complexes are indicated by arrows. Data are representative of experiments performed minimally three times.

Fig. 7

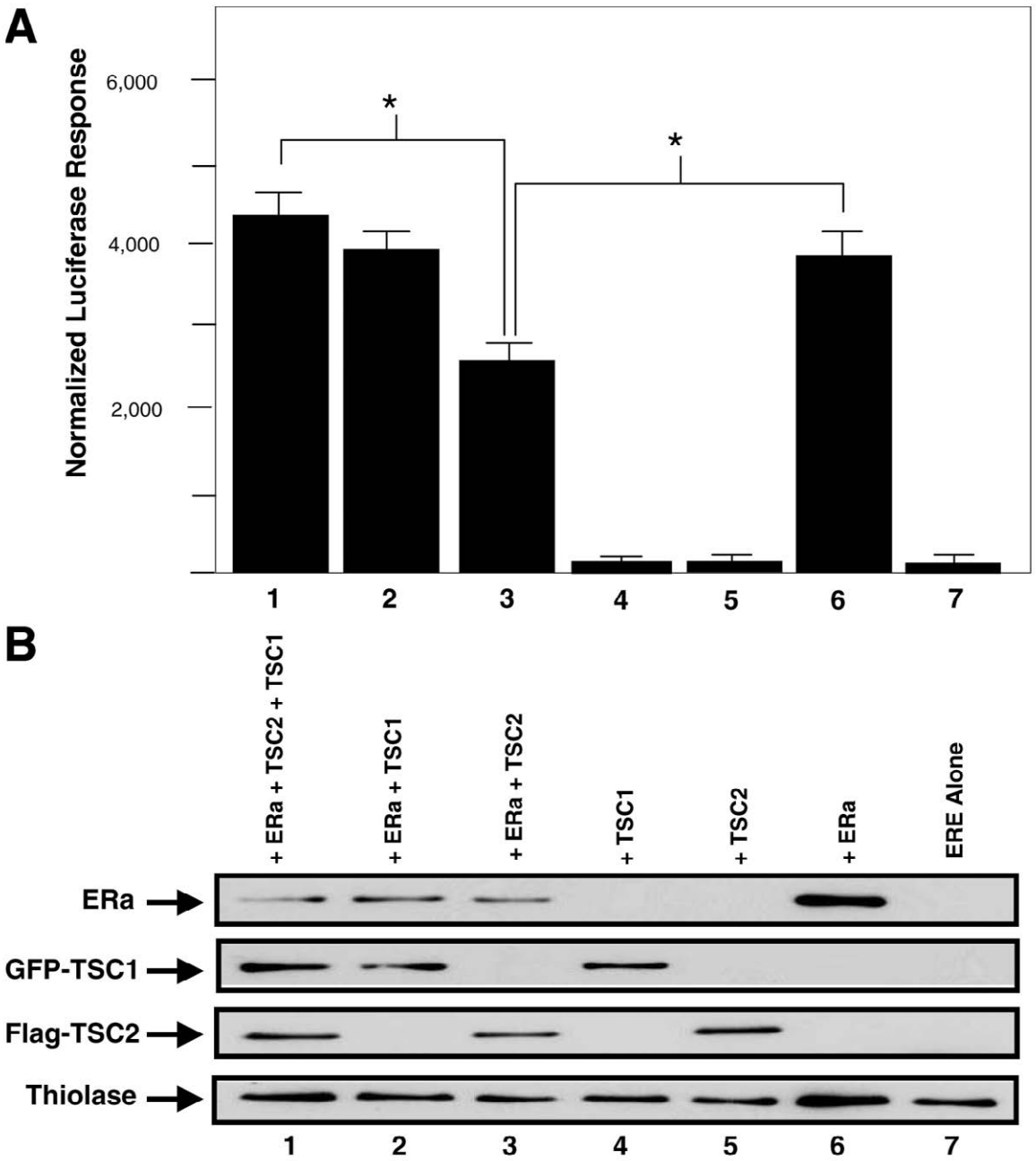

Figure 7. Hamartin expression rescues tuberin's repression of ERα-mediated transcription. ***A***) HEK cells were co-transfected with an ERE-driven luciferase reporter gene and mammalian expression constructs for human ERα, human FLAG-TSC2, and/or human GFP-TSC1 genes. For normalization of transfection efficiency and proliferation, all transfections also included a mammalian expression construct for β-galactosidase (β-Gal). Six hours post-transfection, transfected cells were divided and distributed into a 96-well plate for analysis of luciferase activities **(*A*)** and a 10 cm plate for analysis of protein expression **(*B*)**. ***A***) Cells were incubated for 24 h in the presence of 10 nM 17-β-estradiol. After 24 h, 96-well plates were lysed and analyzed for luciferase and β-Gal activity as described in Materials and Methods. Data are presented as luciferase values normalized to β-Gal activity, and error bars represent standard error of the mean for six samples. *$P < 0.05$ lanes 3 vs. lane 6 (ERα suppression by TSC2) and lane 1 vs. lane 3 (TSC1 rescue of TSC2 repression of ERα). Statistical significance of differences was measured by Student's *t*-test. ***B***) The 10 cm plates were lysed with IP lysis buffer and 50 μg of protein from each plate was analyzed by Western blot probing for the expression of ERα, tuberin (FLAG antibody), hamartin (GFP antibody), and cytoplasmic thiolase. Data are representative of experiments performed minimally three times.