Tuberin Nuclear Localization Can Be Regulated by Phosphorylation of Its Carboxyl Terminus

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Abstract

Tuberin, the tuberous sclerosis 2 (TSC2) gene product, has been identified as a tumor suppressor protein genetically implicated in the pathology of tuberous sclerosis and the female-specific lung disease lymphangioleiomyomatosis. Tuberin and its predominant cytoplasmic binding partner hamartin have been shown to complex with a variety of intracellular signaling regulators and affect the processes of protein translation, cellular proliferation, cellular migration, and cellular transcription. In previous studies, we have presented evidence for tuberin binding to the calcium-dependent intracellular signaling protein calmodulin (CaM), overlap of tuberin CaM binding domain with a binding domain for estrogen receptor α, and the phosphorylation-associated nuclear localization of tuberin. In the study presented here, we expand our findings on the mechanism of tuberin nuclear localization to show that the CaM-estrogen receptor-α binding domain of tuberin can also serve as a tuberin nuclear localization sequence. Furthermore, we identify an Akt/p90 ribosomal S6 kinase-1 phosphorylation site within the carboxyl terminus of tuberin that can regulate tuberin nuclear localization and significantly affect the ability of tuberin to modulate estrogen genomic signaling events. These findings suggest a link between tuberin nuclear localization and a variety of intracellular signaling events that have direct implications with respect to the role of tuberin in the pathology of tuberous sclerosis and lymphangioleiomyomatosis.

(Mol Cancer Res 2006;4(11):885–97)

Introduction

The development of two related proliferation diseases has been genetically linked to mutations in the tuberous sclerosis genes. Tuberous sclerosis complex disease is defined as an autosomal dominantly inherited systemic disorder characterized by a distinct pattern of hamartomas (benign tumors) that develop in a variety of tissues such as the brain, eye, skin, heart, and lung (1, 2). Tuberous sclerosis complex arises from mutations in either the TSC1 gene, which encodes the protein hamartin, or the TSC2 gene, which encodes the protein tuberin. Despite the autosomal dominant mode of transmission, a significant number of sporadically occurring cases have been identified without any prior family history of the disease (3), suggesting a high spontaneous mutation rate in either the TSC1 or TSC2 gene.

Additionally, somatic mutations in these same two genes have been linked to the female-specific lung disease lymphangioleiomyomatosis (4, 5). This disease displays remarkable tissue selectivity, localizing primarily to the lung and secondarily to lymph nodes and kidney. Furthermore, unlike tuberous sclerosis, lymphangioleiomyomatosis has a gender bias, almost exclusively targeting women of childbearing age. Patients with lymphangioleiomyomatosis suffer from a progressive course of dyspnea, pneumothorax, and chylos pleural effusions (6). Exacerbations of lymphangioleiomyomatosis are often seen during pregnancy, and in some cases, the symptoms can be ameliorated with treatments that decrease endogenous estrogen levels, suggesting the involvement of steroid hormones in the pathogenesis of the disease.

Tuberin is a relatively large protein that has been shown to bind or complex with a variety of intracellular signaling proteins (2, 7). Although tuberin has been classically viewed in context with its cytoplasmic binding partner hamartin, it also has been identified as a binding partner for more than 20 other proteins, including isoforms of 14-3-3, protein kinase B (Akt), AMP-activated protein kinase, a variety of cell cycle regulators, several G-proteins, and several nuclear receptors (8). Our previous studies analyzing potential binding partners for tuberin identified, within the extreme carboxyl terminus of tuberin, an overlapping binding domain for the calcium-regulated intracellular signaling protein calmodulin (CaM; ref. 9) and the intracellular receptor for estrogens, estrogen receptor α (ERα; ref. 10). This domain was observed to encompass a six-amino-acid in-frame deletion observed in tuberous sclerosis and lymphangioleiomyomatosis, and also seemed to be linked to the ability of tuberin to modulate steroid/nuclear receptor signaling events (11). These many binding partners suggest that tuberin may function in a variety of intracellular signaling pathways, and, to that end, has been implicated in cellular proliferation events, protein translation, cell migration, neuronal differentiation, and transcriptional regulation (2, 7).

Although classically viewed as an intracellular heterodimer, the many additional binding partners identified for tuberin and hamartin, along with their association with a variety of intracellular signaling pathways, suggest that these proteins may dynamically localize within the cell and function...
independent of each other. This is supported by subcellular localization studies of tuberin-hamartin complexes in human fetuses and tissues (12), biochemical fractionation studies (13-15), and functional studies on tuberin and hamartin-associated signaling pathways (15, 16). Our previously published data provide evidence for the direct involvement of tuberin in nuclear events and, more specifically, in estrogen genomic signaling; here too, the preponderance of evidence suggests that this activity is in the absence of any hamartin interactions (10, 15, 17).

In this report, we present evidence for the presence of a functional nuclear localization sequence (NLS) within the carboxyl terminus of tuberin. This NLS was observed to overlap with tuberin CaM-ERα binding domain. Furthermore, the nuclear localization of tuberin was inhibited by mutations in this domain and the putative tuberin NLS was observed to drive cytoplasmically localized proteins to the nucleus. Finally, phorbol 12-myristate 13-acetate (PMA)–induced phosphorylation of a putative Akt/p90 ribosomal S6 kinase (RSK)-1 phosphorylation site neighboring the NLS was shown to affect nuclear localization of tuberin, and mutations in the RSK1 phosphorylation site were observed to significantly modulate ERα-mediated transcription events. These data strongly support a nuclear role for tuberin and link that role with ERα genomic signaling events.

Results

Tuberin Can Dynamically Localize to the Nucleus

In previous work, we showed that tuberin could transiently localize to the nucleus and that this localization was associated with phosphorylation events within the cell (15). In an extension of those studies, we analyzed the general effects of serum starvation on nuclear localization of overexpressed green fluorescent protein (GFP)–tagged tuberin (Fig. 1). Lymphangioleiomyomatosis cells were plated on glass slides and infected with either a recombinant adenovirus expressing a GFP-tagged TSC2 gene or a control vector expressing GFP alone. Twenty-four hours later, the cells were serum starved for 1 hour, fixed, and fluorescence was visualized by confocal microscopy. As seen here, GFP-tagged tuberin was observed to translocate into and around the nucleus within 1 hour following serum removal. The addition of serum back into this medium drove tuberin back into the cytoplasm in an equally efficient manner (data not shown) and the localization pattern of GFP alone was not altered by the presence or absence of serum. These data suggest that tuberin cellular localization is dynamically regulated by nutrient availability and the subsequent activation and/or inactivation of signal transduction pathways.

A Domain within the Carboxyl Terminus of Tuberin Can Drive Cytoplasmically Localized Proteins to the Nucleus

The rapid movement of tuberin into the nucleus suggests that this property is intrinsic to the tuberin protein. Nuclear localization has been shown to be mediated by members of the karyopherin family (18, 19), predominately karyopherin-α. Karyopherin-α binds to a cluster of basic amino acids on the cargo protein and then forms a heterodimer with importin-β to facilitate the import of cargo into the nucleus (20). A search of the tuberin protein sequence for clusters of basic amino acids identified several candidate NLS moieties. A reasonable candidate NLS was observed to lie between amino acids 1,743 and 1,755 (RLRHIKRLRQRIR). This putative NLS sequence immediately sparked our interest in that it resided in the middle of what we had previously identified as a CaM/ERα binding domain (9, 10), and unpublished studies showed that truncated forms of tuberin that contained this domain seemed to preferentially localize to the nucleus. Furthermore, this domain has been shown to be the site of a six-amino-acid in-frame deletion that has been identified in both tuberous sclerosis and lymphangioleiomyomatosis disease states (21).

To investigate this carboxyl domain as containing a legitimate NLS, a mammalian expression fusion construct was made between a tuberin fragment containing the putative tuberin NLS (amino acids 1,737-1,795) and the β-galactosidase gene. This construct or wild-type β-galactosidase (β-Gal) was transfected into HEK cells and nuclear and cytoplasmic fractions were isolated from these cells and proteins were analyzed in Western blots for the cellular localization of β-Gal (Fig. 2A). As seen here, the presence of the tuberin NLS-containing fragment fused to the β-Gal protein resulted in a substantial increase in nuclear localized β-Gal. These data are consistent with the hypothesis that this tuberin sequence contains an NLS.

Although these data strongly suggest that the putative tuberin NLS is driving nuclear localization, an alternative explanation for some of these data is that the tagged proteins are being anchored to the cytoplasm by tuberin. To investigate this possibility, we generated NLS-containing GFP fusion constructs that were relegated to the carboxyl 73 amino acids of tuberin (GFP-CBD), or this same sequence containing a six-amino-acid in-frame deletion in the putative NLS (GFP-mCBD: ΔRLRHIK of the RLRHIKRLRQRIR sequence; Fig. 2B).
These GFP fusion constructs, along with GFP alone, were transfected into HEK cells; 36 hours posttransfection, the cells were fractionated into nuclear and cytoplasmic fractions and analyzed by Western blot for the localization of GFP and the cytoplasmic protein thiolase. Figure 2C shows that the normal localization pattern of GFP seems to be predominantly cytoplasmic with some nuclear localization. However, the fusion construct containing the putative NLS from tuberin (GFP-CBD) seems to substantially drive GFP localization to the nucleus. Additionally, fusion of the mutant NLS with GFP seems to be less effective in driving GFP nuclear localization than its wild-type counterpart.

In an extension of these studies, COS7 cells transfected with GFP-CBD, GFP-mCBD, or a GFP expression vector were...
analyzed by immunofluorescence (Fig. 2D). As seen in these representative fluorescence micrographs, considerably more nuclear fluorescence is observed in cells transfected with the GFP-tagged tuberin NLS-containing carboxyl-terminal fragment (GFP-CBD) than this same fragment containing the six-amino-acid in-frame deletion (GFP-mCBD). These data support the biochemical fractionation data seen in Fig. 2C and collectively suggest that the nuclear localization potential of tuberin is affected by this putative NLS sequence within the carboxyl terminus of the tuberin protein.

**Coupled Serum Starvation-PMA Activation Stimulates a Tuberin Nuclear Localization Event That Requires Tuberin Carboxyl-Terminal NLS**

The data in Fig. 1 suggest that tuberin transient nuclear localization is coupled to intracellular signaling events induced on serum starvation. To further explore this potential and to more definitively establish the role of tuberin putative NLS in this event, we examined the effects coupled serum starvation-PMA treatment had on the nuclear localization potential of full-length GFP-tagged tuberin (GFP-TSC2) versus GFP fusion mutants of full-length tuberin that either lacked the entire NLS (GFP-TSC2ΔCaM) or contained the six-amino-acid in-frame mutation within the NLS (GFP-TSC2mCBD; Fig. 3A). These constructs were transfected into COS7 cells and subjected to metabolic labeling using [35S]Met/Cys. The metabolically labeled cells were either maintained in normal serum—containing media or subjected to serum starvation followed by 30-minute treatment with the phorbol ester PMA. The cells were subsequently divided into cytoplasmic and nuclear fractions, immunoprecipitated using anti-GFP agarose, and proteins were separated by SDS-PAGE and analyzed by autoradiography. As seen in Fig. 3B, a significant amount of full-length tuberin (GFP-TSC2) translocated to the nucleus on serum starvation and PMA treatment, whereas very little, if any, truncated (GFP-TSC2ΔCaM) and mutant tuberin (GFP-TSC2mCBD) proteins made it to the nucleus.

Corroboration of the above data was seen in immunofluorescence studies on cells transfected with GFP-tagged full-length tuberin and the two tuberin NLS mutant constructs (Fig. 3C). These constructs were transfected into COS7 cells and the cells were either left untreated or serum starved followed by a 30-minute treatment with PMA. As seen in these representative fluorescence micrographs, serum starvation-PMA stimulation resulted in considerably more nuclear fluorescence in cells transfected with the GFP-tagged full-length construct than either of the two mutant constructs. Cumulatively, these data further support the hypothesis that the carboxyl terminus of tuberin contains an NLS and suggest that tuberin nuclear localization may be linked to intracellular signaling pathways.

**Phosphorylation of Ser1798 of the Tuberin Protein Regulates Its Nuclear Localization**

Previously published data have established that posttranslational modification of proteins through phosphorylation/dephosphorylation is the best understood mechanism to regulate nuclear transport (22). Our previous studies strongly supported the hypothesis that phosphorylation events play a role in tuberin nuclear localization (15). Furthermore, published (9) and unpublished studies working with tuberin constructs extensively truncated at its amino terminus suggested that the phosphorylation domain involved in this selective activity most likely resides somewhere in the extreme carboxyl terminus of tuberin. A high stringency computer analysis using the Scansite Motif Scanner1 identified a single Akt kinase phosphorylation site at Ser1798 in the extreme carboxyl terminus of tuberin (Table 1). Based on this information, we investigated the importance of phosphorylation at this site with respect to the nuclear localization potential of tuberin. Flag-tagged TSC2 mammalian expression constructs were created containing the phosphorylation disrupting serine to alanine mutation (Flag-TSC2S1798A) and the phosphomimetic serine to glutamic acid mutation (Flag-TSC2S1798E; Fig. 4A). These constructs, along with the wild-type Flag-TSC2 construct, were transiently transfected into HEK cells. Forty-eight hours after transfection, the cells were lysed and nuclear and cytoplasmic fractions were evaluated in Western blots for tuberin localization (Fig. 4B). As seen here, the glutamic acid phosphomimetic mutant of Ser1798 strongly localized to the nucleus.

To further verify the enhanced nuclear localization of the Flag-TSC2S1798E mutant, immunofluorescence analyses were done (Fig. 4C). COS7 cells were transfected with mammalian expression constructs for Flag-TSC2, Flag-TSC2S1798A, and Flag-TSC2S1798E. Transfected cells were fixed, permeabilized, blocked with normal goat serum, and incubated with an anti-Flag antibody followed by the addition of a FITC-conjugated secondary antibody. Cells were visualized by fluorescence microscopy as described in Materials and Methods. As seen in these representative fluorescence micrographs, cells transfected with Flag-TSC2S1798E displayed substantially stronger nuclear staining than the wild-type TSC2 or the Flag-TSC2S1798A. Cumulatively, these data suggest that phosphorylation of tuberin at Ser1798 can facilitate its nuclear localization.

**Phorbol Esters Stimulate Phosphorylation of Ser1798 and Nuclear Localization of Tuberin**

Ser1798 on tuberin has recently been shown to be a site of phosphorylation by both Akt (23) and phorbol ester–induced activation of p90-RSK1 (24). To investigate whether phorbol ester stimulation of tuberin phosphorylation at Ser1798 has any effect on its ability to localize to the nucleus, HEK cells were infected for 6 hours with a recombinant adenovirus containing an expression plasmid for GFP-TSC2. The infected cells were serum starved for 18 hours, followed by the addition of PMA (100 ng/mL) for 15, 30, 45, and 60 minutes. Cells were divided into cytoplasmic and nuclear fractions and analyzed by Western blot for tuberin localization (Fig. 5A). As seen here, PMA treatment substantially increased nuclear tuberin, with this increase peaking at ~30 minutes, suggesting that PMA activation of p90-RSK1 phosphorylation induces transient nuclear localization of tuberin.

A parallel experiment was done using GFP-TSC2 adenovirus–infected HEK cells that were serum starved and PMA treated for 0 to 60 minutes (Fig. 5B). Total cell lysates were...
generated from treated and untreated cells and assayed for the Raf-mitogen-activated protein kinase/extracellular signal–regulated kinase (ERK) kinase 1/2 signaling pathway components (p42/44 ERK, phospho-p42/44 ERK, p90-RSK, phospho-p90-RSK, Akt, phospho-Akt, and GFP-TSC2). As seen in Fig. 5B, maximal phospho-p42/44 ERK activation (15-minute PMA stimulation) precedes that of phospho-p90-RSK activation (30-minute PMA stimulation), which directly coincides with the maximal influx of tuberin nuclear localization observed in Fig. 5A. Collectively, these data support the hypothesis that activation of the Raf-mitogen-activated protein kinase/ERK kinase 1/2-ERK p42/44 signal transduction pathway can function as a mechanism for induction of tuberin nuclear localization.

To corroborate the above overexpression studies, the potential for nuclear localization of endogenous tuberin in response to PMA stimulation was analyzed. TsA201 cells (an HEK derivative) were incubated overnight in the presence of normal serum–containing media. Cells were either subsequently maintained in normal growth media containing serum, or serum-starved in OptiMem for 18 hours (+). Serum-starved cells were treated with PMA, cytoplasmic and nuclear fractions were generated, and immunoprecipitations were done from these fractions using anti-GFP agarose. The resulting immunoprecipitates were separated by SDS-PAGE and the dried gels were analyzed by autoradiography. Total lysates were generated from these cells and GFP-tagged proteins were immunoprecipitated with anti-GFP agarose to determine total protein expression for each construct.
Table 1. Comparison of the Putative Akt/p90RSK Phosphorylation Site of Tuberin with the Known Substrates Bad and GSK3β

<table>
<thead>
<tr>
<th>Human Tuberin: RKRLI</th>
<th>S1798</th>
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<tbody>
<tr>
<td>Bad: RGRSR</td>
<td>S136</td>
</tr>
<tr>
<td>GSK3β: RRRRTT</td>
<td>S8</td>
</tr>
<tr>
<td>Consensus Seq: RXRx</td>
<td>S/T</td>
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NOTE: The consensus sequences for the substrates are presented and the location of the phosphorylated serine (S) residue within the respective substrates is indicated by number.

Discussion

Accumulated evidence suggests that tuberin plays an important role in a large number of cellular processes including protein translation, cellular migration, cellular proliferation, and transcriptional regulation (2, 7). These processes mandate that tuberin be at least temporally localized to multiple cellular locations. Published immunohistochemical and biochemical analyses further support this hypothesis in that tuberin has been shown to localize to membranes (13, 25), Golgi and other vesicular bodies (26, 27), cytosol (13-15), and nuclei (11, 12). Here we show that tuberin can dynamically localize to the nucleus. These data are consistent with previously published studies showing that tuberin can bind nuclear receptors and modulate the transcription events mediated by these transcription factors (10, 11, 15). Furthermore, these data, for the first time, suggest a link between tuberin intracellular localization and intracellular signaling events. Ser1798 within the highly conserved carboxyl terminus of tuberin was recently shown to interact with and be phosphorylated by both p90-RSK1 (24) and Akt (23). In the case of RSK1, this phosphorylation event was further shown to inhibit the tumor suppressor function of the tuberin-hamartin complex and increase mammalian target of rapamycin signaling to p70-RSK (24), both important mechanisms that function to regulate transnational control within the cell. Data presented here, showing that phosphorylation of Ser1798 also drives tuberin to the nucleus, add a new layer of complexity to these signaling pathways by establishing a point of intersection between RSK1/Akt signaling, ERα/CM signaling, and any other nuclear event that might be affected by the presence of tuberin.

The role of tuberin in nuclear functions is still unclear. The preponderance of immunohistochemical data on mammalian tissues would suggest that tuberin primarily localizes to the cytoplasm in terminally differentiated cells (11, 12, 28).
Alternatively, the lack of any significantly detectable nuclear tuberin in differentiated cells may be a consequence of the transient nature of tuberin nuclear localization or the possibility that antibody epitopes on nuclear localized tuberin may be masked. The extreme carboxyl terminus of tuberin seems to be a potential binding site for several proteins including ERα, CaM, RSK1, and Akt. Furthermore, the RSK1/Akt phosphorylation site at Ser1798 resides in the middle of the epitope for the commercial tuberin antibody most commonly used by tuberin researchers, the tuberin C-20 antibody. Finally, existing data on tuberous sclerosis suggest that cellular proliferation occurs during finite windows of opportunity within specified tissues. A reasonable hypothesis would be that these windows of opportunity correspond to hormonal or growth signaling cues that occur transiently throughout the development of a tissue or organ. This seems to be especially true for the female-specific tuberin-related disease of the lung, lymphangioleiomyomatosis, wherein growth and proliferation of tuberous lesions has been linked to fluctuations in the female sex hormone estrogen. The data presented here also suggest that tuberin dynamically

**FIGURE 4.** Phosphorylation of Ser1798 of the tuberin protein regulates its nuclear localization. 

**A.** Schematic representation of mutations generated at Ser1798 (Flag-TSC2\(^{S1798A}\) and Flag-TSC2\(^{S1798E}\)) of the tuberin protein. 

**B.** HEK cells were transiently transfected with mammalian expression constructs for Flag-TSC2, Flag-TSC2\(^{S1798A}\), and Flag-TSC2\(^{S1798E}\). Forty-eight hours after transfection, these cells were harvested and divided into cytoplasmic and nuclear fractions. Equivalent amounts of the cytoplasmic and nuclear lysates were separated on SDS-PAGE gels and probed in a Western blot for expression of tuberin (top). The integrity of the fractionation was evaluated by reprobing blots for the expression of the cytoplasmic protein thiolase (bottom). 

**C.** Mammalian expression constructs for Flag-TSC2, Flag-TSC2\(^{S1798A}\), and Flag-TSC2\(^{S1798E}\) were transiently transfected into COS7 cells using the Superfect transfection protocol. Forty-eight hours posttransfection, cells were harvested, plated into chamber slides, and probed for expression of tuberin with an anti-Flag antibody and a FITC-conjugated secondary antibody. Cells were stained with 4',6-diamidino-2-phenylindole to identify nuclei. Cells were visualized by fluorescence microscopy and adjustments for brightness and contrast were applied uniformly to the entire field. Cells depicted are representative of the majority of transfected cells. Representative of experiments done at least thrice.
localizes to the nucleus, and the cis-domain within tuberin is responsible for this nuclear localization is part of the cis-domain responsible for both ERα and CaM binding.

Data presented in this article provide evidence for tuberin localization to the nucleus, preferentially when it is phosphorylated at Ser1798. These findings would, at first glance, seem to be paradoxical with respect to our earlier published data that showed that the general kinase inhibitor staurosporine stimulated tuberin nuclear localization (15). Both sets of data are highly reproducible, and we can only offer several reasonable speculations to explain these differences. The first and most logical is the fact that staurosporine is a fairly broad-spectrum kinase inhibitor and could be modulating a number of other signaling pathways that might affect tuberin nuclear localization; or the reverse is also possible, wherein although a variety of tuberin phosphorylation pathways are affected by staurosporine, those specific signaling pathways phosphorylating Ser1798 are not sensitive to staurosporine. The latter is supported in our earlier studies by the observation that tuberin phosphorylation is not completely inhibited by staurosporine (15). Alternatively, as speculated above, the immunoreactivity with the epitope for the commercial C-20 antibody used in the previous studies might be affected by the addition of a phosphate group to Ser1798 sitting in the middle of this epitope.

**FIGURE 5.** Phorbol esters stimulate nuclear localization of tuberin. A. HEK cells were infected with an adenovirus containing a mammalian expression construct for GFP-TSC2 or GFP alone. Eighteen hours postinfection, cells were serum fed (Ø) or serum starved for 18 hours followed by the addition of PMA for 15, 30, 45, and 60 minutes as indicated. Cytoplasmic and nuclear fractions were isolated and equivalent amounts of each fraction were run on SDS-PAGE gels. Western blotted, and probed with a GFP antibody to detect GFP-TSC2 (top). To evaluate nuclear and cytoplasmic fractionation, the resulting blots were reprobed for cytoplasmic thiolase (bottom). B. HEK cells were infected with recombinant adenoviruses expressing GFP-TSC2 or GFP alone. Eighteen hours postinfection, cells were serum starved for 18 hours followed by the addition of PMA for 15, 30, 45, and 60 minutes, or refed with serum (SR) for 60 minutes. Cells were lysed and equivalent amounts of each lysate were separated by SDS-PAGE and Western blotted, probed for expression of phospho-ERK, total ERK, phospho-p90-RSK, total p90-RSK, phospho-Akt, total Akt, GFP (for tuberin), and thiolase as indicated. C. Cultured TsA201 cells were either maintained in serum containing media (NT), serum starved for 18 hours (SS), serum starved followed by a time course of PMA treatment for 15, 30, 45, and 60 minutes, or treated with vehicle alone (DMSO). Cells were fractionated into cytoplasmic and nuclear fractions. An equivalent aliquot of each fraction was removed and assayed by Western blot analysis for the cytoplasmic control protein thiolase (bottom). The remainder of the fraction was subjected to an immunoprecipitation with a tuberin-specific antibody. The immunoprecipitate was separated by SDS-PAGE, Western blotted, and the blot was probed with a mouse monoclonal tuberin antibody (top). D. TsA201 cells were transfected with either Flag-TSC2<sup>1798A</sup> (rows 1 and 2) or Flag-TSC2 (rows 3 and 4) mammalian expression constructs. Cells transfected with Flag-TSC2<sup>1798A</sup> were either maintained in serum containing media (NT), or serum starved for 18 hours followed by a time course treatment with PMA for 15, 30, 45, and 60 minutes, or treated with DMSO vehicle alone for 30 minutes (row 1). Cells transfected with Flag-TSC2 were either maintained in normal serum—containing media (NT) or serum starved followed by 30-minute treatment with PMA (row 3). Cytoplasmic and nuclear fractions were generated from all cell populations and an equivalent amount of each fraction was removed and evaluated by Western blot for the cytoplasmic control protein thiolase (rows 2 and 4). Anti-Flag agarose was used to isolate Flag-tagged proteins from the remaining lysates. These were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and analyzed by Western blot, probed with a tuberin-specific antibody. Representative of experiments done at least thrice.
and thus masking nuclear tuberin detection. The studies in this article attempted to avoid that potential complication by using tuberin expression constructs tagged at their amino terminus with a Flag epitope.

Recent studies have shown that, should tuberin be able to translocate to the nucleus, it could have significant effects on both specific transcription and perhaps more global transcription events. York et al. (10) showed that tuberin can bind directly with ERαs and can block ERα-DNA binding. Furthermore, CaM, another reported binding partner of tuberin that binds in an overlapping domain with ERαs (9), has been shown to also play a critical role in ERα-DNA binding (29, 30) as well as the binding of other transcription factors (31, 32). Here we show that mutations in tuberin CaM/ERα binding domain can strongly affect the ability of tuberin to translocate to the nucleus, and that a RSK1 kinase domain at Ser1798 adjacent to the tuberin CaM/ERα binding domain can regulate tuberin nuclear localization as well as the ability of tuberin to modulate ERα-mediated transcription. Although intriguing and provocative, especially with respect to diseases like lymphangioleiomyomatosis and perhaps some breast cancers, it should be noted that these transcription analyses were done with reconstituted cell lines and using overexpression scenarios; therefore, further studies will be required to establish a true physiologic and disease relevance.

Data presented in this article identify two novel conditions that regulate tuberin nuclear localization: PMA stimulation and serum starvation. It has been well established that tuberin plays a central role in modulating energetic signals through both the AMP-activated protein kinase (33) and mammalian target of rapamycin (34) pathways; but at first glance, it would seem to be somewhat counterintuitive that mitogen-induced cellular proliferation would also drive tuberin nuclear localization. Although these two conditions seem to be opposite fates from a cellular perspective, they do share one biochemical feature that may explain the need for transiently localized nuclear tuberin. It is well established that both nutrient depletion (35) and cellular mitosis (36) result in transient suppression of cap-dependent protein translation. The well-established role of tuberin in regulation of translation would support the hypothesis that transient nuclear tuberin could function to sequester either free tuberin or, more probably, the nuclearlocalized mammalian target of rapamycin complexes (37, 38) away from the translational machinery. Therefore, transient nuclear localization of tuberin could serve as the universal mechanism for regulating cap-dependent translation during the seemingly diametrically opposed cellular processes of nutrient depletion and mitosis. In this regard, we have initiated a series of ongoing studies to investigate tuberin nuclear binding partners.

Data presented here circumstantially suggest that Ser1798 on tuberin preferentially serves as a RSK1 phosphorylation site. Although previously published data show that this site can also be very weakly phosphorylated by Akt in vitro (23), Roux et al. (24) have clearly shown that Ser1798 of the tuberin protein is a specific in vitro target of p90-RSK on activation with PMA, and that when analyzed with respect to Akt, this site is highly preferred by p90-RSK, whereas sites at Ser939 and Thr1462 are preferred targets for Akt. This analysis would fit with the studies herein, where the data would predict that serum starvation mediates the phosphorylation of Ser1798. Although the mechanism responsible for this kinase activity
needs investigation, one possible candidate kinase would be protein kinase Cε, which has recently been shown to localize in the Golgi, endoplasmic reticulum, and nuclear envelope and to translocate to the nuclear envelope on PMA activation and serum starvation (39).

Although the focus of the studies presented here was on the mechanism and consequence of tuberin nuclear localization, any discussion of tuberin signaling cannot be made without consideration of its predominant cytoplasmic binding partner, hamartin. Like tuberin, hamartin has also been reported to localize to the nucleus (40). If hamartin localizes to the nucleus, a reasonable question would be, do these proteins function within the nucleus as a heterodimer or independently? At present, there is no definitive answer to this question, but circumstantial data from our studies presented here and those of other labs would suggest nuclear tuberin and hamartin function independently of each other. These data include (a) immunohistochemical studies on fetal tissues that suggest that tuberin and hamartin do not colocalize to overlapping regions of the nucleus (28); (b) a variety of studies that show phosphorylation of tuberin often disrupts tuberin-hamartin interactions (41, 42); and (c) studies presented here that show phosphorylation of tuberin can drive its nuclear localization. In spite of this evidence, it is clear that further research will be required to definitively establish the functions hamartin and tuberin might be playing in the nucleus.

Mutations in the TSC2 gene have been genetically mapped to tuberous sclerosis and the female-specific lung cancer lymphangioleiomyomatosis (3, 43). More than 600 mutations in the tuberin gene have been mapped for these diseases, and although they seem to occur throughout the protein, a strong argument can be made that most of these mutations ultimately result in a loss of function at the carboxyl terminus of tuberin (44). One such reported mutation, a six-amino-acid in-frame deletion that occurs in its carboxyl terminus, is localized in the middle of a putative CaM and ERα binding domain on tuberin and has been reported to disrupt tuberin-CaM binding and tuberin-ERα interactions and to modulate nuclear receptor-mediated gene expression events (9, 10). Here we show that this putative CaM-ERα binding domain can also serve as a NLS for tuberin and that the aforementioned six-amino-acid in-frame deletion residing within this putative tuberin NLS impairs tuberin nuclear localization. The relevance of these findings to the pathology of lymphangioleiomyomatosis and tuberous sclerosis is currently under investigation.

In summary, studies in this article have shown that tuberin can dynamically localize to the nucleus, have identified the domain within the carboxyl terminus of tuberin that is responsible for nuclear localization, and have shown that this event can be regulated through a RSK1 phosphorylation site in the extreme carboxyl terminus of tuberin. Furthermore, these studies suggest that Ser1798 at the carboxyl terminus of tuberin could serve as a direct link for crosstalk between RSK1 (mitogen-activated protein kinase), CaM (calcium), and ERα (estrogen) signaling pathways. Finally, these studies help to shed light on the conundrum imposed by a loss of functional tuberin in diseases like tuberous sclerosis, wherein it has been observed that this loss can disrupt a variety of intracellular signaling pathways and that these tissues can be affected by a variety of hormone-induced signaling events.

**Materials and Methods**

**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). Antibodies used for the detection of tuberin, β-Gal, and GFP were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and those used to detect the Flag epitope from Sigma. Antibodies used for the detection of ERK p42/44, phospho-ERK, p90-RSK1, phospho-p90-RSK1, Akt, and phospho-Akt were obtained from Cell Signaling Technology, Inc. (Beverly, MA). Mouse monoclonal antibody for the detection of ERα was purchased from Lab Vision Corp. (Fremont, CA). The mouse monoclonal tuberin antibody was obtained from Zymed Laboratories (South San Francisco, CA). Secondary antibodies were purchased from both Cell Signaling Technology and LI-COR, Inc. (Lincoln, NE), and the antibody for the detection of thiourea was a generous gift from Dr. Sidney Whiteheart (University of Kentucky, Lexington, KY). The bicinchoninic acid protein concentration kit was purchased from Pierce Biotechnology, Inc. (Rockford, IL).

**Plasmid Constructs**

The construction of the mammalian expression constructs for tuberin (pED-TSC2), tuberin with a six-amino-acid in-frame deletion (Δ1746-1752) in its CaM binding domain (pED-TSC2mCBD), GFP-TSC2, human ERα (pRSV-hERα), pCMV-β-Gal, and the pBL-ERE-K-luc luciferase reporter construct were as previously described (9, 11, 15). For the GFP-CBD fusion constructs, TSC2 insert cDNAs were excised from the parental pED-TSC2 and pED-TSC2mCBD vectors by digesting with XbaI/EcoRV and were subcloned into XbaI/blunted-ended BglII sites of the pEGFP-C1 mammalian expression vector (BD Bioscience, Palo Alto, CA). The fusion constructs for GFP-TSC2mCBD and GFP-TSC2ΔCaM were generated by excising the original pED-TSC2mCBD and pED-TSC2ΔCaM constructs (15) with XbaI/SalI, and these were subcloned into the unique XbaI/SalI sites of the pEGFP-C1 vector system. The mammalian expression construct in which the tuberin NLS was fused to the β-galactosidase gene (pCMV-NLS-β-Gal) was created by using primers (forward, AGGCCCTAGGCGCTTCCGCTGGCC-CACCTCA; reverse, AGCGCTGAGAATCCAAAGGTGGATT-GCCCGGCT) to PCR isolate a 174-bp fragment from the carboxyl end of the TSC2 coding region sequence (bp 5,212-5,306). This sequence was digested with the restriction enzyme BsrUI and subcloned into the unique BsrUI site of the pCMV-β-Gal vector (Clontech, Palo Alto, CA), inserting the NLS between amino acids 102 and 103 of the β-Gal protein. An amino-terminal Flag-tagged TSC2 mammalian expression construct was created using primers (forward, GCCGCCCTAGGCGCTTCCGCTGGCC-CACCTCA; reverse, TCCAGGGCGCCCTCCGGGCTGACACGATCC) to PCR a 311-bp fragment containing the sequence....
amino terminus of the TSC2 gene fused to a Flag epitope. This fragment was digested with SalI and NotI and subcloned into SalI/NotI-digested pED-TSC2. The Flag-TSC2 1798 serine to alanine (pED-Flag-TSC2 S1798A) and serine to glutamic acid (pED-Flag-TSC2 S1798E) mutants were made by using primers (S1798A reverse, GATCTCTAGAGCCGGCTACTACA-CAATCGGTGAAGTCCCTCCAGGCATGCATGAGGCC; S1798E reverse, GATCTCTAGAGCCGGCCTCACAATCGGTGAAGTCCCTCCAGGCATGACATGAGGCC; and common forward primer, CACCGATATCCACACAAATCGGTGAAGTCCCTCCAGGCATGATGAGGCC) to PCR point mutations into Ser1798 of the tuberin protein. These PCR products were digested with the restriction enzymes EcoRV and XbaI and subcloned into EcoRV/XbaI-digested pED-Flag-TSC2 mammalian expression construct. The integrity of all constructs was verified by DNA sequencing and routine molecular technologies were used throughout these constructions (45).

**Cellular Fractionation**

To divide cell populations into nuclear and cytoplasmic fractions, standard differential centrifugation techniques were employed as we have previously described (15). Briefly, cells were lysed in a buffer containing 1% (v/v) NP40, passed through a 20-gauge needle, brought to a final concentration of 5 mmol/L MgCl2, and nuclei were pelleted by centrifugation at 600 × g for 5 minutes at 4°C. The supernatant (cytoplasmic fraction) was collected and centrifuged at 16,000 × g for 10 minutes at 4°C to eliminate nuclear carry over. The nuclear pellet was washed thrice in lysis buffer, examined for integrity and purity of the nuclei by trypan blue exclusion, lysed by bringing the solution to 0.3% SDS, and the DNA was sheared by digestion (5 minutes, 4°C) with Benzonase nuclease (Novagen, Inc., La Jolla, CA). Both the cytoplasmic and nuclear lysates were stored at −80°C until use. Protein concentration determinations were established using the commercially available Bradford reagent (Bio-Rad, Inc., Hercules, CA) and assayed essentially as described by the manufacturer.

**Signal Transduction Assays**

To investigate the effect of PMA stimulation on tuberin intracellular localization, HEK cells were infected with an adenovirus containing an expression construct for GFP-tuberin or GFP alone. Infected cells were either maintained in serum-free media or serum starved followed by the addition of PMA (100 ng/mL) for 15, 30, 45, and 60 minutes. Cells were washed twice in 1× PBS and removed by trypsinization. Cells were further washed to remove residual trypsin and fractionated as described above into cytoplasmic and nuclear fractions. An equivalent amount of each fraction was separated on SDS-PAGE, transferred to a nitrocellulose membrane, and analyzed by Western blot by probing with a mouse monoclonal GFP antibody. Additionally, duplicate samples were run and assayed in Western blots for expression of the cytoplasmic control antibody. Likewise, to evaluate the effect of PMA stimulation, equivalent aliquots of each fraction were also assayed by Western blot analyses for p42/44 ERK, p42/44 ERK and phospho-ERK, phospho-RSK, total RSK, phospho-Akt, total Akt, GFP-tuberin, and thiola.

To study the effect of PMA stimulation of endogenous tuberin localization, Tsa201 (an HEK derivative) cells were either maintained in serum-containing media, serum starved alone for 18 hours, serum starved for 18 hours followed by a time course treatment with PMA (100 ng/mL) for 15, 30, 45, and 60 minutes, or treated for 30 minutes with DMSO vehicle. Cells were collected and separated into nuclear and cytoplasmic fractions as described above. Each fraction was assayed for total protein concentration by Bradford and an equivalent aliquot of each fraction from each independent treatment was removed and assayed by Western blot for expression of the cytoplasmic protein thiola. The remainder of each fraction was subjected to an immunoprecipitation by incubating lysates with a tuberin-specific antibody for 2 hours at 4°C followed by the addition of a 50:50 mixture of protein A/G-Sepharose. The bound immunoprecipitate was washed extensively with IP lysis buffer [50 mmol/L Tris·HCl (pH 7.5), 150 mmol/L NaCl, 1% NP40, and 0.1% SDS]. The immunoprecipitate was separated by SDS-PAGE, transferred to nitrocellulose membrane, and Western blotted, probing with a mouse monoclonal anti-tuberin antibody (Zymed).

**Western Blot**

Western blot analyses were done as we have previously described (15). Briefly, proteins separated by SDS-PAGE were Western blotted onto nitrocellulose membranes, blocked in PBS containing 1% casein, incubated with primary antibody diluted in PBS containing 1% casein and 0.1% Tween 20, washed in TBST [20 mmol/L Tris·HCl (pH 7.6), 137 mmol/L NaCl, 0.1% Tween 20], incubated with appropriately conjugated secondary antibodies, and analyzed either by direct IR fluorescence on an Odyssey imaging system or reacted with enhanced chemiluminescence reagents (Pierce) and detected on X-ray film by autoradiography.

**Analysis of Transfected Cells with Fluorescently Tagged Proteins**

Cytologic analysis of cells transfected with fluorescently tagged proteins or proteins to be studied by fluorescence was done as previously described (9). Transfected COS7 cells were grown on coverslips, washed with 1× PBS, and fixed for 15 minutes in 4% paraformaldehyde. Fluorescence microscopy of these cells was done as previously described (21) using a Zeiss Axiovert 200M microscope equipped with epifluorescence with a Hamamatsu Orca ER high-resolution digital imaging system.

Lymphangioleiomyomatosis cells for confocal microscopy studies were plated at a density of ~10,000 per well of a chamber slide (Nalge Nunc International, Rochester, NY) and infected with an adenovirus expressing GFP-TSC2 as described above. Cells were serum starved for 1 hour and rinsed thrice with HBS/ Ca2+. Cells were subsequently washed in HBS supplemented with 1 mmol/L CaCl2 and 1 mmol/L MgCl2 and fixed in 4% paraformaldehyde for 15 minutes at room temperature. Cellular localization of GFP-TSC2 was visualized on a Leica TCS confocal microscope system at ×40 magnification. Adjustment of brightness and contrast on digitally acquired images was applied uniformly to the entire field.
**Immunohistochemical Analyses**

Immunohistochemical studies were done essentially as previously described (46). Briefly, 48 hours posttransfection, cells were washed, trypsinized, and assayed for integrity and number by trypan blue exclusion. Viable cells were replated at a cell density of ~10,000 per well of an eight-well chamber slide. Cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. Cells were blocked in 1% normal goat serum followed by the addition of anti-Flag antibody (20 μg/mL). Cells were washed extensively with 1× PBS containing 0.05% Tween 20, followed by the addition of an antirabbit FITC-conjugated secondary antibody. Slides were washed extensively with 1× PBS containing 0.05% Tween 20 and mounted using Vectashield containing 4',6-diamidino-2-phenylindole to facilitate nuclear staining. Cells were visualized by fluorescence microscopy as described above. Adjustments for brightness and contrast were applied uniformly to the entire field.

**Metabolic Labeling**

To investigate the effect mutations in tuberin NLS have on tuberin nuclear localization potential, COS7 cells were transfected with mammalian expression constructs for GFP, GFP-TSC2, GFP-TSC2ΔCaM, or GFP-TSC2mCBD using Lipofectamine Plus (Invitrogen, Inc., Carlsbad, CA). Twenty-four hours posttransfection, cells were washed twice with 1× PBS and subjected to a starvation in cysteine- and methionine-deficient media for 30 minutes at 37°C. Cells were washed with PBS followed by metabolic labeling for 3 hours with 100 μCi/mL of tran-[35S]l-[35S]methionine and cysteine. This was followed by a 1-hour chase with normal DMEM. Metabolically labeled cells were washed with 1× PBS and placed in OptiMem reduced serum media overnight or maintained in normal DMEM with 10% fetal bovine serum. Serum-deprived cells were treated with PMA (100 ng/mL) for 30 minutes. Cells were washed twice with 1× PBS and removed by trypsinization. Cells were pelleted by centrifugation at 1,500 × g for 5 minutes and subsequently washed twice with 1× PBS to remove any residual trypsin. Cells were processed as described above into cytoplasmic and nuclear fractions. The resulting fractions were brought to 1× radioimmunoprecipitation assay buffer [100 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1% sodium deoxycholate, 0.1% Triton X-100, 0.1% SDS, 1× Roche protease inhibitor cocktail, 1× Calbiochem phosphatase inhibitor cocktail] and GFP-tagged proteins were isolated from the fractions by incubating them with anti-GFP agarose (Vector Laboratories, Inc., Burlingame, CA) for 1 hour at 4°C. The resulting bead-bound protein fractions were washed twice in radioimmunoprecipitation assay buffer supplemented with 0.3 mol/L NaCl twice in radioimmunoprecipitation assay buffer containing 0.15 mol/L NaCl, and once in SDS wash buffer [50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 2.5 mmol/L EDTA]. Samples were separated by SDS-PAGE, dried, and placed on a Phospho-Imager screen. Samples were analyzed using the Typhoon Imaging system (Amersham, Piscataway, NJ).

**Mammalian Transcription Assays**

**In vitro** transcription assays using a standard calcium phosphate precipitation protocol were done as we have previously described (11). HEK cells were cotransfected with 4 μg of pCMV-Flag-TSC2, Flag-TSC2ΔCaM, or Flag-TSC2mCBD along with 2 μg of human pRSV-ERα, 5 μg of pCMV-β-Gal, and 5 μg of a pBL-ERE-κ-Luc luciferase reporter plasmid. The total amount of plasmid per 10-cm plate was brought to 20 μg using pGEM4 plasmid DNA. Transfections were incubated with the calcium-phosphate/DNA precipitates for 6 hours and washed extensively with 1× PBS. Cells were removed by trypsinization, a portion of the cells were replated (5 × 10^5 per well) into six wells per 10-cm plate of a 96-well plate (in the presence and absence of 10 mmol/L 17β-estradiol), and the remainder of the cells were replated into a 10-cm plate to be used for total protein expression analyses. Twenty-four hours after transfection, induced luciferase and normalizing β-Gal activities were determined as previously described (11). Activity was expressed as the average of sextuplet luciferase activities normalized to the average β-Gal rate [average luciferase response / (average β-Gal response per minute)] for corresponding wells. Data were graphed as the SE for the six wells analyzed per transfection. Cells replated posttransfection were washed twice in 1× PBS, lysed by the addition of Flag-IP buffer [50 mmol/L Tris-HCl (pH 7.5), 150 mmol/L NaCl, 1% NP40, 0.1% SDS and 1× Roche protease inhibitor cocktail], protein concentrations were determined, and an equivalent portion of each lysate was run on an SDS-PAGE gel and evaluated by Western blot for the expression levels of ERα and thiolase. Flag-tagged proteins were extracted from the remainder of the lysate by incubating it with anti-Flag agarose (Sigma). The agarose beads were extensively washed and bound proteins were eluted into SDS-PAGE sample buffer, separated by SDS-PAGE, transferred to a nitrocellulose membrane and analyzed by Western blot, probing with an anti-tuberin antibody.

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Tuberin Nuclear Localization Can Be Regulated by Phosphorylation of Its Carboxyl Terminus

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