Phosphorylation of Tuberin as a Novel Mechanism for Somatic Inactivation of the Tuberous Sclerosis Complex Proteins in Brain Lesions

Sangyeul Han,1 Túlio M. Santos,1 Ana Puga,1 Jenn Roy,2 Elizabeth A. Thiele,3 Mia McCollin,3 Anat Stemmer-Rachamimov,2 and Vijaya Ramesh1

1Molecular Neurogenetics Unit, 2Molecular Neuro-Oncology, and 3Department of Neurology, Harvard Medical School/Massachusetts General Hospital, Charlestown, Massachusetts

Abstract

Tuberous sclerosis complex (TSC) is an autosomal dominant, multisystem disorder characterized by the presence of hamartomas in brain, kidney, heart, lung, and skin. Common TSC-associated brain lesions include cortical tubers, subependymal nodules, and subependymal giant cell astrocytomas (SEGA; Ref. 1). Neurologic complications include seizures, mental retardation, and autism. The disease is caused by mutations in tumor suppressor genes TSC1 or TSC2 encoding hamartin and tuberin, respectively (2, 3). Hamartin and tuberin associate in vivo forming a complex with other proteins.

Introduction

Tuberous sclerosis complex (TSC) is an autosomal dominant, multisystem disorder characterized by the presence of hamartomas in many organs. Although tuberous sclerosis complex is a tumor suppressor gene syndrome with classic “second hits” detectable in renal tumors, conventional genetic analysis has not revealed somatic inactivation of the second allele in the majority of human brain lesions. We demonstrate a novel mechanism of post-translational inactivation of the TSC2 protein, tuberin, by physiologically inappropriate phosphorylation, which is specific to tuberous sclerosis complex-associated brain lesions. Additional analysis shows that tissue specificity is due to abnormal activation of the Akt and nitogen-activated protein kinase pathways in brain but not in renal tumors. These results have widespread implications for understanding the tissue specificity of tumor suppressor gene phenotypes.

Results

Expression of Hamartin and Tuberin in Tubers and SEGAs. Our earlier findings that somatic mutations with subsequent loss of the wild-type allele in TSC1 or TSC2 are not common in the CNS lesions of patients with TSC suggested the possibility of haplo-insufficiency...
in these lesions (19). To address this further, we determined whether tuberin and hamartin were expressed in these tumors. Expression of tuberin and hamartin was examined in cortical tubers from a patient with TSC who had been identified previously as having a germ-line frameshift mutation in the TSC2 gene but lacking a second somatic mutation including loss of heterozygosity in tubers (19). Immunohistochemistry was performed using antihamartin (HF6) and antituberin antibody (TSDF), which specifically recognize hamartin and tuberin, respectively. A strong cytoplasmic staining pattern was observed in both giant/balloon cells (Fig. 1A) and maloriented, abnormal neurons (Fig. 1B), the target cell types of the cortical tubers, indicating that tuberin could be detected in these tubers. Furthermore, expression of hamartin also was detected in giant cells of the tubers (Fig. 1D). These results support those of our previous genetic studies demonstrating the loss of loss of heterozygosity or other inactivating somatic mutations in TSC2 and TSC1 in these cortical tubers (19). In addition, analysis of a SEGA (xT3515) from another unrelated patient with TSC showed robust expression of tuberin in tumor cells (Fig. 1C).

**Activation of the mTOR/S6K Pathway in Tubers and SEGAs.** Hamartin and tuberin inhibit the mTOR and S6K pathways. In renal lesions of patients with TSC and in Eker rats, in which biallelic inactivation of TSC2 and TSC1 is common, mTOR and S6K are constitutively activated in vivo (6, 20). Therefore, we raised the question as to whether tuberin or hamartin, expressed in tubers and SEGAs, may be adequate or inadequate for the regulation of the activity of a downstream target like mTOR. Western blot analysis was performed on lysates from three SEGAs from which sufficient frozen material was available to determine the status of mTOR, phospho-mTOR, S6K, and phospho-S6K levels. Phospho-mTOR and phospho-S6K were detected in vivo in SEGAs but not in normal human brain used as a control. Total mTOR and total S6K levels were seen in SEGAs and in brain (Fig. 2A). Additionally, expression of ribosomal protein S6, an effector of mTOR, and phospho-S6 (P-S6) was analyzed. Compared with normal brain, SEGA xT3515 showed marked phosphorylation of S6. Similarly, compared with normal kidney, two angiomylipoma samples from TSC patient 9 described previously (19) revealed increased P-S6 levels (Fig. 2B). The cytoplasm of giant cells from the tubers of TSC patient 9 (19), and giant cells and dysmorphic neurons from another unrelated patient with TSC displayed strong positive staining for P-S6 (Fig. 2, C and D). Immunohistochemical staining demonstrated intense, uniform expression of P-S6 (Ser235/236) in SEGA xT3515 (Fig. 2E). As described in a recent study (6), immunohistochemistry of angiomylipoma showed positive P-S6 staining (Fig. 2F). DU145, a PTEN-positive cell line, was used as a negative control for P-S6 (Fig. 2G). Together, these results indicate that levels of hamartin and tuberin expressed in the CNS lesions were either incapable of suppressing mTOR or they were not functional.

**Tuberin Is Phosphorylated and Inactivated in SEGAs and Tumors.** It is well known that activation of the phosphatidylinositol 3’-kinase (PI3K)-regulated serine-threonine kinase Akt/protein kinase B phosphorylates tuberin, resulting in inactivation of tuberin in cultured cells. Therefore, we raised the question as to whether tuberin expressed in tubers and SEGAs is phosphorylated and inactivated. To test this hypothesis, we examined phospho-tuberin expression in tubers and SEGAs using a phospho-specific tuberin antibody against Thr1462 (pT1462). An earlier study showed constitutive phosphorylation of tuberin at T1462 in a prostate cancer cell line (PC3) that is mutated for PTEN (11). Employing two prostate-derived tumor cell lines as controls, DU145 (PTEN positive) and PC3 (PTEN negative), we examined the status of tuberin and phospho-tuberin by immunoblots in three SEGAs along with normal brain. Under serum-deprived conditions tuberin expression was seen in both prostate cancer lines; however, unlike DU145, constitutive phosphorylation of tuberin at T1462 was noted only in the PC3 cell line, which is consistent with an earlier report (11). Interestingly, in two SEGAs (xT3515 and xT819), constitutive phosphorylation of tuberin was seen along with tuberin expression. Expression of tuberin was very weak in SEGA xT1614. In the normal brain, tuberin was expressed but not phosphorylated (Fig. 3, A, top). In two angiomylipoma samples tested, tuberin was not expressed, supporting biallelic inactivation in these lesions (Fig. 3A, bottom). The pT1462 antibody for phosphotuberin was used in im-

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**Fig. 1.** Expression of tuberin and hamartin in tubers and a subependymal giant cell astrocytoma (SEGA) from patients with tuberous sclerosis complex. A and B, tubers stained with tuberin antibody TSDF. A, positive cytoplasmic staining of a multinucleated giant cell (arrow; bar = 80 μm). B, staining in dysplastic neurons (arrows; bar = 160 μm). C, diffuse cytoplasmic staining of cells in the SEGA with TSDF antibody (bar = 160 μm). D, tuber stained with hamartin antibody HF6. Arrow indicates a positively stained abnormal giant neuron (bar = 80 μm).
munohistochemical staining using PC3 and DU145 cell pellets as positive and negative controls (Fig. 3B). Phospho-tuberin staining was intense in SEGA xT3515 (Fig. 3C) and in balloon cells from a tuber (patient 9; Fig. 3D, arrow). These results not only demonstrate for the first time tuberin phosphorylation in vivo in CNS lesions but also show a clear difference between hamartomas associated with the kidney and those associated with the brain.

**Activation of Akt and PDK1 in SEGAs.** Intrigued by the phosphorylation state of tuberin in SEGAs and tubers, we hypothesized that tuberin could be phosphorylated by activation of the PI3K/Akt pathway in CNS-derived hamartomas. Akt is activated by phospholipid binding and phosphorylation at Thr308 and Ser473 by PDK1 (23). Therefore, we investigated the state of phosphorylated Akt using an antibody that recognizes phospho-Akt at Ser473 (P-S473). Compared with normal brain, in all three of the SEGAs Akt was activated (Fig. 4A). We next examined the expression of phospho-PDK1 (Ser241) to observe whether PDK1 was also activated in these SEGAs. Phosphorylation of Ser241 on PDK1 is essential for the activity of PDK1 (24). As shown in Fig. 4A, phospho-PDK1 was expressed in all three of the SEGAs but not in the normal brain, suggesting activation of PDK1 exclusively in SEGAs (Fig. 4A). In addition, two angiomyolipomas derived from TSC patient 9 (19) did not show activation of Akt. As expected, PC3 (PTEN negative) cells expressed phospho-Akt (Ser473) owing to lack of PTEN, leading to

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**Fig. 2.** Expression of activated mammalian target of rapamycin (mTOR) effectors in tubers and subependymal giant cell astrocytomas (SEGAs). A, expression of phospho-mTOR (Ser2448) and phospho-S6K (Thr389/421) in three different SEGAs (xT3515, xT1614, and xT819) and unrelated normal brain. B, Western blot analysis showing expression of phospho-S6K (Ser235/236). Left panel, SEGA (xT3515); right panel, angiomyolipomas. C–G, immunohistochemical staining showing phospho-S6 (Ser235/236) expression. C, a giant cell of tuber (bar = 80 μm). D, dysplastic neurons in a tuber (bar = 160 μm). E, SEGA (xT3515, bar = 80 μm). F, angiomyolipoma from a patient with tuberous sclerosis complex (TSC) showing positive staining (bar = 160 μm). G, DU145, a PTEN+ cell line showing negative staining (bar = 80 μm).

**Fig. 3.** Phosphotuberin (Thr1462) expression in tubers and subependymal giant cell astrocytomas (SEGAs). A, Western blot analysis showing phospho-tuberin expression in SEGAs. Lysates of serum-starved PC3 (PTEN−) and DU145 (PTEN+) were used as a positive and negative control, respectively, along with lysates of SEGAs and angiomyolipomas using either antituberin or antiphosphotuberin (Thr1462) antibodies. B–D, immunohistochemical staining with phosphotuberin (Thr1462) antibody. B, DU145 and PC3 cells (bar = 80 μm). C, SEGA (xT3515; bar = 160 μm). D, tuber (bar = 80 μm, arrow indicates a giant cell with a positive staining in cytoplasm).
Akt activation, and DU145 cells showed lack of Akt activation (Fig. 4B). Our findings indicate that both Akt and PDK1 are activated in SEGAs but not in angiomyolipomas. Because Akt and PDK are upstream of tuberin, these results support the constitutive phosphorylation and inactivation of tuberin in SEGAs.

These observations raised the question as to which signals may be responsible for the activation of Akt/PDK1 in these lesions. One possibility could be the somatic inactivation of PTEN because mutations in the PTEN tumor suppressor lead to aberrant activation of the PI3K-Akt pathway (25). Germ-line mutations in PTEN are known to occur in Cowden’s disease and Bannayan-Zonana syndrome, both of which share with TSC the occurrence of hamartomas (26, 27). Therefore, we analyzed DNA samples from three SEGAs and a normal brain for somatic mutations in all nine of the exons of PTEN by single-strand conformation polymorphism screening. We did not find any evidence for PTEN mutation (data not shown). Furthermore, the state of total PTEN and phospho-PTEN were similar between SEGAs and normal brain (Fig. 4C). These observations suggest that Akt/PDK1 pathway is not activated by somatic inactivation of PTEN in SEGAs.

Expression of Phospho-Mek1/2 and Phospho-Erk1/2 in SEGAs. Recent reports indicate that the p38-activated kinase MK2 and protein kinase C/mitogen-activated protein kinase (MAPK) signaling can lead to phosphorylation and inactivation of tuberin in a PI3K-independent manner (28, 29). These reports prompted us to investigate whether MAPK signaling is activated constitutively in SEGAs. Interestingly, both phospho-Erk1/2 and phospho-Mek1/2 were expressed in all three of the SEGAs but not in normal brain, angiomyolipoma samples, and normal kidney (Fig. 4D), indicating that the MAPK/extracellular signal-regulated kinase signaling cascade is abnormally activated in these SEGAs. These results suggest that MAPK pathway may also be involved in regulation of tuberin phosphorylation in vivo.

Discussion

Our earlier work on somatic mutations of TSC lesions documented that although kidney lesions may frequently show biallelic inactivation of TSC genes, this phenomenon may not be common in the CNS lesions. Based on this assumption, we suggested that haploid levels of tuberin or hamartin expressed in the CNS lesions may be inadequate to regulate the activity of downstream target proteins that may play a role in growth stimulation. Alternatively, expression of the wild-type protein may be “turned off” or reduced as a result of epigenetic events or cooperating mutations in genes other than TSC (19). In the present study we show that, although expressed in SEGAs and tubers, tuberin and hamartin are unable to regulate the activity of downstream targets like mTOR and S6K. Furthermore, we show that tuberin is phosphorylated in CNS lesions, resulting in inactivation. Thus, in CNS lesions, somatic inactivation of the tuberin-hamartin complex appears to involve a mechanism distinct from the conventional biallelic inactivation seen in kidney lesions.

Our results also reveal that Akt and PDK1 are activated in CNS lesions, resulting in tuberin phosphorylation. Akt activation also appears to be distinct in CNS lesions, as kidney tumors derived from the Eker rat (6) as well as two angiomyolipoma lesions tested in this study did not reveal this phenomenon. Other studies have shown suppression of insulin-induced Akt activation in Tsc2−/− MEF lines, which was thought to result from a negative feedback loop from mTOR/S6K to the PI3K signaling pathway (5, 7). The possibility that Akt activation in SEGAs could result from somatic inactivation or haplinsufficiency of PTEN has been ruled out, as we did not detect PTEN mutations in the SEGAs. In addition, we show that the MAPK/extracellular signal-regulated kinase pathway is activated only in the TSC brain lesions but not in angiomyolipomas, normal kidney, or normal brain (Fig. 4D). In this context, it is interesting to note the recent observation that protein kinase C/MAPK signaling leads to phosphorylation of tuberin in cultured cells at sites that overlap with and are distinct from Akt-phosphorylation sites (29). These studies provide evidence that cell signaling that converges through the PI3K and protein kinase C/MAPK pathways inactivates the tuberin-hamartin complex as a result of tuberin phosphorylation. Furthermore, during the preparation of the present article, a report appeared demonstrating a high level of activated MAPK expression by immunohistochemistry in a SEGA (30), consistent with our immunoblotting data. In Tsc2−/− MEF cell line MAPK was not activated (5). From these results, it is clear that tissue-specific regulation plays a role in TSC lesion development.

The mechanism that activates the Akt and MAPK pathways in CNS lesions has not been determined. One possibility is that the microenvironment, which is unique to these lesions, results in secretion of growth factors or other neurotrophic factors. Growth factors secreted by adjacent interstitial cells or TSC lesions may activate the Akt and MAPK pathways, leading to tuberin phosphorylation. Angiogenic factors such as basic fibroblast growth factor, platelet-derived endothelial cell growth factor, vascular endothelial growth factor, and angiogenin are detected in the interstitial cells and vascular cells of the angiomyolipomas from patients with TSC (31). Loss of Tsc1 and Tsc2 in mice leads to secretion of vascular endothelial growth factor, which is dependent on mTOR signaling (32). Furthermore, a very recent study using Tsc2 null fibroblasts show that tuberin, by virtue of its ability to inhibit mTOR, down-regulates the hypoxia-inducible factor, which is a transcription factor. The increase in hypoxia-inducible factor levels in Tsc2−/− cells activates a variety of genes implicated in tumorigenesis, including vascular endothelial growth factor (33). Both vascular endothelial growth factor and basic fibroblast growth factor are known to activate PI3K/Akt pathway in a protein kinase C and extracellular signal-regulated kinase-dependent manner (34). Another candidate may be the neurotrophins (NT), such as NT-3 and NT-4, and their receptors, tyrosine kinase receptor B and tyrosine kinase receptor C. Endogenously produced neurotrophins such as NT-3 and brain-derived neurotrophic factor signal via tyrosine kinase receptors
to activate the PI3K/Akt and MAPK/extracellular signal-regulated kinase pathways to regulate survival and differentiation, respectively, of cortical progenitors (35). Interestingly, expression of NT-4 and tyrosine kinase receptor C mRNA is increased in dysplastic neurons and giant cells of TSC-associated cortical tubers (36), which may play a role in activation of the Akt and MAPK pathways in SEGAs and tubers. However, the involvement of other genetic or epigenetic events that may be responsible for discrete activation of Akt and MAPK in CNS lesions cannot be ruled out. Although the paucity of tissue samples available from patients with TSC limits the approaches necessary to address the tissue-specific regulation, efforts aimed at banking various hamartomatous lesions ultimately will aid in a better understanding of in vivo mechanism(s) that may contribute to tumorgenesis in various settings.

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REFERENCES


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