Tuberin Phosphorylation Regulates Its Interaction with Hamartin

TWO PROTEINS INVOLVED IN TUBEROUS SCLEROSIS*

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Hamartin and tuberin are products of the tumor suppressor genes, TSC1 and TSC2, respectively. When mutated, a characteristic spectrum of tumor-like growths develop resulting in the syndrome of tuberous sclerosis complex. The phenotypes associated with TSC1 and TSC2 mutations are largely indistinguishable suggesting a common biochemical pathway. Indeed, hamartin and tuberin have been shown to interact stably in vitro and in vivo. Factors that regulate their interaction are likely critical to the understanding of disease pathogenesis. In this study, we showed that tuberin is phosphorylated at serine and tyrosine residues in response to serum and other factors, and it undergoes serial phosphorylation that can be detected by differences in electrophoretic mobilities. A disease-related TSC2 mutation (Y1571H) nearly abolished tuberin phosphorylation when stimulated with pervanadate. Expression of this mutant tuberin caused a marked reduction in TSC1-TSC2 interaction compared with wild-type protein and significantly curtailed the growth inhibitory effects of tuberin when overexpressed in COS1 cells, consistent with a loss of function mutation. Examination of a second pathologic mutation, P1675L, revealed a similar relationship between limited phosphorylation and reduced interaction with hamartin. Our data show for the first time that 1) tuberin is phosphorylated at tyrosine and serine residues, 2) TSC1-TSC2 interaction is regulated by tuberin phosphorylation, and 3) defective phosphorylation of tuberin is associated with loss of its tumor suppressor activity. These findings suggest that phosphorylation may be a key regulatory mechanism controlling TSC1-TSC2 function.

The autosomal dominant syndrome of tuberous sclerosis complex (TSC) is a disorder classified as a phakomatosis and is characterized by the development of hamartomatous lesions that are patchy in distribution in multiple organ systems (1). The common clinical manifestations stem from growths in the brain (cortical tubers), kidney (angiomyolipoma and cysts), heart (rhabdomyomas), and skin (2). As such, it commonly results in epilepsy, mental retardation, renal complications, and premature deaths. Although pathologically diverse, the lesions in TSC share common features of excessive proliferation, abnormal differentiation, and aberrant migration. Genetic studies of families have successfully identified two genetic loci that are responsible for this disease (3, 4). Within the last few years, the two genes, TSC1 and TSC2, have been positioned-ally cloned, allowing investigations that will lead to the understanding of the molecular basis of TSC.

The function of these genes has been partially elucidated to date. There is evidence to suggest that the TSC2 product, tuberin, suppresses tumorigenicity (5, 6), controls cell cycle (7–9), affects normal brain development (10), exhibits in vitro Rap1 GTPase-activating protein activity (11), modulates transcription in vitro (12), interacts with the TSC1 protein, hamartin (13, 14), and participates in vesicular trafficking (15). In addition, hamartin was shown to interact with ezrin-radixin-moesin and regulate cell adhesion (16). However, there is no unifying concept of how these two genes may influence these processes. Further, there has not been any study addressing the regulation of hamartin and tuberin in the context of their physiologic role as tumor suppressor genes.

Among the various properties of these two proteins, their ability to interact and to form a stable complex has been the most consistent finding (13, 14). Their interaction fits well with the concept that the gene products function along a common biologic pathway. Thus, mutation of either gene can give rise to a similar clinical phenotype. This led to the hypothesis that hamartin and tuberin may function as a complex, and factors regulating their interaction will be important in understanding their physiologic roles. In this study, we discovered that tuberin undergoes reversible phosphorylation at serine and tyrosine residues that can be detected by mobility shifts on SDS-PAGE gels in response to serum and other stimuli. Two disease-related missense mutations of TSC2 gave rise to proteins that were defective in their ability to be phosphorylated. As such, they were unable to interact efficiently with hamartin, thus abolishing the tumor suppressor activity of the complex. These findings provide novel insights into the mechanisms of TSC1-TSC2 gene functional regulation.

EXPRESSITIONAL PROCEDURES

Materials—HeLa, COS1, and NIH3T3 cells were obtained from the American Type Culture Collection (Manassas, VA). EEF126–4, EEF 126–8, and LEF8 cells were derived as described previously (5, 15). All cell culture reagents, LipofectAMINE, and Plus reagent were purchased from Life Technologies, Inc. Thin layer cellulose plates were obtained from Whatman (Clifton, NJ), and PP1 (catalytic subunit) phosphatases were obtained from Calbiochem. Horseradish peroxidase-conjugated donkey

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The abbreviations used are: TSC, tuberous sclerosis complex; PV, permeability voltage; LAR, leukocyte antigen-related protein-tyrosine phosphatase; PP1, protein phosphatase 1; FBS, fetal bovine serum; PVDF, polyvinylidene fluoride; PAGE, polyacrylamide gel electrophoresis; AEBSF, 4-(2-aminoethyl)benzenesulfonylfluoride, HCl.

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anti-rabbit and anti-mouse antibodies and ECL reagent were purchased from Amersham Pharmacia Biotech. Anti-phosphotyrosine (PY99) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The tuberin (15) and hamartin antibodies were purified using the EcosoPac serum IgG purification columns from Bio-Rad. Polyclonal anti-phosphotyrosine was raised in rabbits against a 15-mer amino acid peptide (KHDELPRWRKRL (238–252), conjugated to bovine serum albumin. The antisera was affinity-purified using the Pierce Aminolink Plus immobilization kit. Immobilon-P PVDF membranes were purchased from Millipore (Bedford, MA). Other chemicals were purchased from Sigma. Wild-type TSC1 in pcDNA3 (Invitrogen, Carlsbad, CA) was a kind gift from Elizabeth Henske (Fox Chase Cancer Center, Philadelphia, PA) (14).

**Site-directed Mutagenesis**—Polymerase chain reaction-based mutagenesis was performed with the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The previously described rat wild-type TSC2 in pBS plasmid was used as the template (5). The oligonucleotide primers designed to introduce the mutations were as follows (the base change is underlined): forward primer Y1571H, 5'-GCTGGCTCTTATAGGACACGATGCTG-3'; reverse primer Y1571H, 5'-CAGAAACCTGTGCTGCTAAGAGCCATGC-3'; forward primer P1675L, 5'-CCATGGATCATCACTCAGCTGATTATAAATGC-3'; reverse primer P1675L, 5'-GACCTAGATGCATCCAGCAGTGTTAGATCATCACTGAT-3'. After sequencing, Y1571H and P1675L were subcloned into wild-type TSC2 by digesting with AoeI and HindIII. The entire TSC2 sequence was then subcloned into the NcoI and XhoI sites of pcDNA3 (Invitrogen, Carlsbad, CA).

**Cell Culture and Treatments**—EEF126–4 and EEF126–8 cells were cultured as described previously (5, 15). HeLa, NIH3T3, and COS1 cells were cultured according to ATCC protocol. The TSC1 and TSC2 plasmids were transfected into 60–80% confluent COS1 cells using LipofectAMINE as described by the manufacturer. 48 h post-transfection, cells were cultured as shown in figures. A stock solution of pervanadate (PV) was prepared fresh each time by mixing equal volumes of 0.1 M H$_2$O$_2$ and 0.1 M Na$_3$VO$_4$. The stimulation reactions were stopped by centrifugation at 15,000 × g for 10 min. The supernatant was used for experiments.

**Immunoprecipitation and Immunoblotting**—Aliquots containing equivalent amounts of cellular protein were incubated for 2 h at 4 °C with 1 µl of either IgG-purified L3–2 (TSC2) or 4050 (TSC1) antibodies. 20 microliters of 1:1 (v/v) of protein A-Sepharose in harvest buffer was added to each tube and incubated overnight. Immunoprecipitates were washed three times with harvest buffer and resuspended in 30 µl of 1.5× sample buffer. For phosphatase treatment, immunoprecipitates were washed with harvest buffer containing no EGTA or sodium vanadate, incubated at 30 °C for 30 min on a rotator, and stopped by adding 4× sample buffer. Cell extracts or immunoprecipitates were electrophoresed on a 7% low (30:0.4) bisacrylamide SDS-PAGE gel and transferred to PVDF membrane for Western blotting. Tuberin was visualized using polyclonal IgG-purified anti-tuberin (L5–2) (15) at a dilution of 1:500. The hamartin antibody was used at a dilution of 1:100, and both were followed by horseradish peroxidase-conjugated anti-rabbit secondary antibodies at a dilution of 1:5000. Antibody complexes were visualized by chemiluminescent detection using ECL reagent.

**32P Labeling and Phosphoamino Acid Analysis**—COS1 cells in 60-mm dishes were transfected as above. Cells were grown in Dulbecco’s modified Eagle’s medium with 0.1% FBS overnight, washed with phosphate-free Dulbecco’s modified Eagle’s medium, and then incubated with 0.5 µCi of orthophosphate in 2 ml of phosphate-free Dulbecco’s modified Eagle’s medium with 0.1% FBS for 6 h. 10% FBS and pervanadate (0.1 µM final concentration) were added to the cells 30 min prior to the end of labeling. The cells were harvested, and ~50 µg of total protein was immunoprecipitated as above. The immunoprecipitates were resolved by SDS-PAGE and transferred to PVDF membrane, and the 32P-labeled tuberin was visualized by autoradiography.

Phosphorylation analysis was performed as described previously (17, 18). Briefly, the individual 32P-labeled tuberin bands were excised and subjected to acid hydrolysis in 6 N HCl at 110 °C for 1 h. The hydrolyzed protein was transferred to a new microcentrifuge tube, lyophilized, and dissolved in 12 µl of H$_2$O. Two-dimensional TLC was performed by spotting 1/12 of the hydrolyzed protein plus 1 µl of a 1 mg/ml mix of phosphoserine, phosphothreonine, and phosphotyrosine into the corner of a TLC plate. The first-dimension separation was performed by chromatography in ethanol/glacial acetic acid/water (8:3:4, v/v/v) for 40 min at room temperature. Separation in the second dimension was performed by chromatography in isobutyl alcohol/formic acid/water (8:3:4, v/v/v) for 40 min at room temperature. Phosphoamino acids were visualized by spraying with a solution of 0.5% ninhydrin in 0.5% ace tone and heating at 80 °C for 5 min. Labeled amino acids were visualized by autoradiography.

**Cell Proliferation Assay**—COS1 cells were transfected as above. 24 h post-transfection, cells were trypsinized and counted using a hemocytometer. The cells were then spun down, washed with phosphate-buffered saline, and lysed by addition of harvest buffer as above. Protein assays were performed using the Pierce BCA protein assay kit.

**RESULTS**

**Tuberin Undergoes Gel Mobility Shifts According to Phosphorylation State**—It has been observed previously that polyclonal anti-tuberin antibodies can detect multiple bands at the expected molecular mass range of 180–195 kDa on Western blotting (11). This has been attributed to the existence of known TSC2 splice variants. In this study, we examined whether the variation in tuberin mobility is related to a change in phosphorylation states. Endogenous tuberin was examined in cell lines derived from the Eker rat, which carries a germline mutation of TSC2 (1.1A). The 126–4 rat embryo fibroblasts contain wild-type tuberin that migrated as a broad band, and compared with a faster migrating TSC2 protein from the LEF8 cells derived from a renal tumor. The TSC2 gene in the latter cells does not exhibit abnormal splicing of exons 25 or 31, and the remaining TSC2 coding regions did not show any deletion (data not shown). This led us to believe that other modifications such as phosphorylation could be responsible.

To determine whether the mobility difference was because of phosphorylation, cells were treated with 100 µM PV, a protein-tyrosine phosphatase inhibitor, and kinase activator. Following 30 min of treatment, wild-type tuberin in 126–4 cells showed a shift toward the slower migrating forms, whereas the tuberin from LEF8 cells showed minimal mobility change (Fig. 1A). The observed mobility shift of the wild-type tuberin was also noted in other cell lines with the exception of COS1 cells, where the majority of the endogenous tuberin migrates slower constitutively (Fig. 1B). Wild-type tuberin overexpressed in COS1

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cells, however, exhibited a band shift in response to pervanadate (Fig. 1C). This, in part, was associated with tyrosine phosphorylation as shown by specific immunoreactivity to the anti-phosphotyrosine antibody, PY99. Further, the slower migration of the TSC2 band caused by pervanadate can be reversed by treatment with various phosphatases (Fig. 1C). Exposure of wild-type tuberin to λ phosphatase dramatically enhanced its mobility and eliminated immunoreactivity to PY99. Recombinant PP1 (catalytic subunit), which possesses serine/threonine and some tyrosine phosphatase activities (19), caused a similar shift in mobility, as well as loss of PY99 immunoreactivity. Treatment with a tyrosine phosphatase, LAR (catalytic domain), eliminated the PY99 signal but did not affect mobility of the tuberin band. These observations are consistent with multiple phosphorylation events affecting tuberin mobility. Although other modifications such as ubiquitination can affect electrophoretic mobility, such changes are not reversible by phosphatase treatment (20). We conclude that the frequently observed “doublets” and band shifts of tuberin on Western blotting can be accounted for by various states of protein phosphorylation.

To determine whether tuberin phosphorylation is part of the physiologic response to serum and other stimuli, electrophoretic migration of tuberin was examined following exposure to various factors. Among the treatments, FBS (10%) and anisomycin (25 μg/ml) produced a band shift, whereas platelet-derived growth factor-BB (20 ng/ml) and forskolin (10 μM) did not have a detectable effect on the migration of tuberin from TSC2-transfected COS1 cells (Fig. 2A). Endogenous tuberin also responded in a similar fashion. Upon serum starvation, tuberin in NIH3T3 cells shifted toward the dephosphorylated state whereas serum-fed cells maintained a level of phosphorylation in between the starved and pervanadate-treated states (Fig. 2B). In contrast, tuberin in the tumor-derived LEF8 cells did not respond to the treatments and remained in a mobility state that was faster than that seen with wild-type tuberin during serum starvation. Taken together, the data showed that tuberin undergoes reversible phosphorylation in response to multiple extracellular signals, and its phosphorylation state can be detected by electrophoretic migration. Also, one might infer that the tuberin from LEF8 cells could be defective in its ability to be phosphorylated. Interestingly, hamartin did not show a significant change in band migration under the same conditions (Fig. 2B, lower panel), future studies will address the role of phosphorylation for hamartin.

Tuberin Is Phosphorylated at Tyrosine and Serine Residues—To determine which amino acids are phosphorylated, tuberin overexpressed in COS1 cells (starved or stimulated with FBS/PV) was labeled in vivo with [32P]orthophosphate followed by immunoprecipitation with anti-tuberin antibodies.

The immunoprecipitates were resolved by SDS-PAGE, transferred onto PVDF membranes, and examined by autoradiography. The bands corresponding to phosphorylated tuberin were excised from the membrane, digested, and subjected to phosphoamino acid analysis and Cerenkov counting. As shown in Fig. 3A, LAR phosphatase reduced total phosphorylation to 82% of the PV-treated level while maintaining much of the tyrosine phosphorylation despite the loss of PY99 signal on Western blotting. Both PP1 and λ phosphatases decreased phosphorylation of tuberin to 14 and 11% of the pervanadate-treated control, respectively. Overall, the amount of [32P]orthophosphate incorporation correlated well with tuberin mobility. When the samples were analyzed by two-dimensional TLC, it was clearly shown that tuberin was phosphorylated on serine and tyrosine but not threonine residues (Fig. 3A).

Disease-causing Mutations of TSC2 Affect Its Phosphorylation—To evaluate the biologic significance of TSC2 phosphorylation, two naturally occurring TSC2 mutants were examined. The first mutant was found in the LEF8 cells, an Eker-derived renal tumor cell line that has not undergone loss of heterozygosity at the TSC2 locus. Sequence analysis of the TSC2 coding regions confirmed a single base pair substitution (T4800C, rat) (data not shown). This results in a non-conservative amino acid change, from tyrosine to histidine (Y1571H, human; Y1573H, rat). This tyrosine lies within the putative Rap1 GTPase-activating protein homology domain of TSC2 and is highly conserved in evolution.

To examine the phosphorylation properties of the Y1571H mutant, an expression construct containing this change was made by site-directed mutagenesis and was overexpressed in COS1 cells. Western blotting confirmed the increased mobility of the expressed mutant protein and the minimal shift in response to PV treatment, similar to that seen in the parental LEF8 cells (data not shown). Based on the amount of [32P]orthophosphate incorporation, the Y1571H mutant labeled very minimally after FCS/PV treatment, compared with wild-type tuberin (Fig. 3B). Further, TLC analysis showed that both tyrosine and serine phosphorylation were significantly reduced. This is consistent with a model in which tyrosine 1571 is a phosphorylation site that may be required for serial phosphorylation of both serine and tyrosine residues.

A second TSC2 missense mutation that has been recurrently identified in patients with tuberous sclerosis was also exam-
TSC2 Phosphorylation

FIG. 4. Effects of TSC2 mutants on the interaction of tuberin with hamartin. A, endogenous proteins were immunoprecipitated (IP) with either anti-tuberin (top) or anti-hamartin (bottom) antibodies. The LEF8 cells contain the Y1571H mutant allele and express both tuberin and hamartin. Negative control, 126–8 (TSC2−/−); positive controls, 126–4 (TSC2+/−) and NIH3T3 (TSC2−/−). C, control; S, starved. B, extracts of COS1 cells overexpressing TSC2 mutants, Y1571H (Y/H), P1675L (P/L), or wild-type (WT) TSC2 were subjected to immunoprecipitation (IP) with anti-tuberin (left) or anti-hamartin (right) antibodies. Relative amount of co-precipitated proteins is shown by immunoblotting with anti-tuberin (top) and anti-hamartin (bottom) antibodies.

The P1675L mutation was isolated from 10 unrelated TSC individuals, and it lies within the rapamycin-binding domain (21). The expression construct containing this amino acid change was generated by site-directed mutagenesis and confirmed by direct sequencing. Overexpression of the P1675L construct produced a stable protein that migrated faster than the wild-type tuberin, similar to that seen with the Y1571H mutant (Fig. 4B). The P1675L mutant tuberin did not respond to pervanadate treatment, and phosphoamino acid analysis confirmed minimal incorporation of [32P]orthophosphate affecting both the serine and tyrosine residues (Fig. 3B). Our data suggest that tuberin phosphorylation is affected by disease-causing mutations.

Effects of Phosphorylation on Hamartin-Tuberin Interaction—Substantial evidence indicates that tuberin and hamartin form a stable complex in vivo (13, 14). We hypothesized that phosphorylation of tuberin may play a role in regulating its interaction with hamartin. Co-immunoprecipitation experiments were performed on endogenous and overexpressed, exogenous TSC1 and TSC2 proteins. As expected, the wild-type hamartin and tuberin in EEF126–4 and NIH3T3 fibroblasts formed a stable complex as they were able to be immunoprecipitated by both anti-tuberin and anti-hamartin antibodies (Fig. 4A). However, the mutant form of tuberin found in tumor cells, LEF8, did not interact with hamartin. The lack of co-immunoprecipitation of the endogenous proteins was not because of the absence of hamartin. In addition, when the mutant Y1571H and wild-type TSC1 were transiently overexpressed in COS1 cells, only a fraction of the two proteins were recovered as a complex, compared with the wild-type TSC2 (Fig. 4B). Under conditions of protein overexpression (>20-fold increase), some degree of interaction was retained by the mutant product. When the expression constructs were transiently transfected into tuberin-deficient rat embryonic fibroblasts, EEF126–8 cells, transgene expression was modest (~2-fold), and only the wild-type tuberin, not the Y1571H mutant, was shown to interact with hamartin (data not shown). We also tested the ability of the P1675L mutant to interact with hamartin when expressed in COS1 cells. Again, the amount of complex formation relative to the expression levels of the individual proteins was significantly less for the mutant, compared with the wild-type tuberin (Fig. 4B).

Effects of Mutant Tuberin on Proliferation—Previous experiments have shown that wild-type tuberin exerts growth inhibitory effects when overexpressed in cells, transiently or stably (5). We tested the biologic activity of the Y1571H mutant in transient transfection of COS1 cells. 24 h after transfection, the cell number and protein concentration were measured as an index of cell proliferation (Fig. 5). In accordance with previous studies, cells overexpressing wild-type tuberin showed a reduction of 49% in cell number and a drop of 50% in protein concentration, compared with the vector control. In contrast, the Y1571H mutant induced only a modest reduction of 20% in cell number and 27% in protein concentration. In both cases, the exogenous tuberin was expressed to nearly equal levels of at least 20-fold over the endogenous levels. The same result in cell number reduction was observed when the experiment was repeated in NIH3T3 cells (data not shown).

DISCUSSION

Tuberous sclerosis is an example of the “two genes, one disease” paradigm where mutation of either one of its two responsible loci, TSC1 and TSC2, will result in the expression of a similar phenotype. Evidence from genetic and biochemical analyses is congruent with the concept of functional cooperativity of the two gene products, hamartin and tuberin. However, the biologic significance of their interaction and its regulation have not been reported. In this study, we demonstrated that tuberin is phosphorylated at serine and tyrosine residues, and this process is altered by the two missense mutations examined. One consequence of the hypophosphorylated mutant forms of tuberin is the reduction in their interaction with hamartin and correspondingly, the loss of the growth inhibitory function of tuberin. Our data support the notion that regulation of the tuberin-hamartin functional complex can lead to the initiation of disease. We also showed that various phosphorylation states of tuberin can be easily detected by their different electrophoretic mobilities.

The regulation of tuberin phosphorylation is likely complex and has not been fully elucidated in this study. The wild-type TSC2 product undergoes reversible phosphorylation in response to physiologic stimuli, such as serum. Based on phos-
TSC2 Phosphorylation

phosphoamino acid analysis and electrophoretic mobility shifts, there exists multiple sites involving serine and tyrosine residues. Assuming that each mobility shift represents a different phosphorylation state, we can deduce a minimum of four levels of TSC2 phosphorylation beyond the basal state, but mapping of specific phosphorylation sites awaits further studies.

Of the two mutants described, the effect of Y1571H on tuberin phosphorylation strongly suggests that the Y1571 can itself be phosphorylated. Review of other pathologic missense TSC2 mutations has identified four additional tyrosine or serine residues that may be relevant to its phosphorylation (21). Based on \(^{32}\)P labeling, the effect of the P1675L mutant on reducing tuberin phosphorylation can be interpreted as more complete than the Y1571H mutation. The disruption of the proline residue is predicted to have profound effects on the structural conformation of the protein and consequently, is likely to abolish interaction with the appropriate kinase(s).

This site resides in the rabaptin5-binding domain (15) and represents a common amino acid altered by naturally occurring mutations; it was reported in 10 independent cases of tuberous sclerosis (21). Our findings lead us to believe that tuberin phosphorylation is targeted by natural mutations and is important to its function.

Although direct interaction between TSC1 and TSC2 proteins has been confirmed by yeast two-hybrid analyses and co-immunoprecipitation studies (13, 14), the binding domains have not been completely elucidated. Reports have suggested that the N terminus of tuberin is essential for hamartin interaction (13, 22). Here, we showed that phosphorylation sites outside the putative interaction domains are also critical to hamartin-tuberin interaction. Because there exists a high degree of concordance between interaction and phosphorylation in the mutants studied in this report, it is difficult to isolate the effects of interaction on phosphorylation. The fact that wild-type tuberin overexpressed alone can undergo phosphorylation in response to pervanadate treatment to the same degree as when it is co-expressed with hamartin would strongly suggest that tuberin phosphorylation is not dependent on its interaction with hamartin.\(^2\)

The concept of phosphorylation-regulated protein interaction has been noted in diverse signaling pathways. Our characterization of tuberin phosphorylation and its effects on hamartin interaction provides new insights into the regulation of its tumor suppressor function. Analyses of the missense mutations support the biologic significance of TSC2 phosphorylation. Further understanding of the pathways that modify its phosphorylation state will provide opportunities to manipulate its function exogenously as therapy for patients with tuberous sclerosis.

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