Phosphatidylinositol 3-Kinase/Akt Pathway Regulates Tuberous Sclerosis Tumor Suppressor Complex by Phosphorylation of Tuberin*

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Han C. Dan‡§, Mei Sun‡§, Lin Yang‡§, Richard I. Feldman¶, Xue-Mei Sui‡, Chien Chen Ou‡, Mark Neillist***, Raymond S. Yeung||, Dicky J. J. Halley***, Santo V. Nicosia‡, Warren J. Pledger‡, and Jin Q. Cheng‡‡

From the ‡Department of Pathology, Molecular Oncology, and Drug Discovery Programs, University of South Florida College of Medicine, H. Lee Moffitt Cancer Center & Research Institute, Tampa, Florida 33612, the ¶Cancer Research Department, Berlex Biosciences, Richmond, California 94804, the ||University of Washington Medical Center, Seattle, Washington 98195, and the **Department of Clinical Genetics, Erasmus University, 3015 GE Rotterdam, The Netherlands

Normal cellular functions of hamartin and tuberin, encoded by the TSC1 and TSC2 tumor suppressor genes, are closely related to their direct interactions. However, the regulation of the hamartin-tuberin complex in the context of the physiologic role as tumor suppressor genes has not been documented. Here we show that Akt physically interacts with and phosphorylates tuberin, which is mediated by the PI3K/Akt pathway but not by the MAPK pathway. As a result, the regulation of hamartin and tuberin in the context of physiologic role as tumor suppressor genes has not been documented.

This article has been withdrawn by the authors. The same data were used to represent different experimental conditions. Specifically, the tuberin panel from transfection of pcDNA3 from Fig. 3C was reused as the hamartin panel from transfection of TSC2-7A from Fig. 3D, and the tuberin panel from transfection of Myr-Akt from Fig. 3C was reused as the TSC2-7D panel from transfection of TSC2-7D-Xpress in Fig. 3D. The authors state that the overall conclusions of this work are not affected.

EXPERIMENTAL PROCEDURES

Plasmids—The cytomegalovirus-based expression constructs encoding wild type, constitutively active, and dominant negative Akt, Myc-TSC1, and TSC2-Xpress have been described (3, 17). TSC2-7A and Akt7/kinesin 2 and Akt6/FKBPs, all of which are activated by growth factors and insulin in a PI3K-dependent manner and are inhibited by PTEN tumor suppressor (14). Akt regulates a wide spectrum of cell functions, including cell survival, cell growth, differentiation, angiogenesis, and glucose metabolism, through phosphorylation of a number of proteins that contain the RXRXXS/T motif (14–16).

Here we show that Akt physically interacts with and phosphorylates tuberin, leading to degradation of the hamartin-tuberin complex and p27kip1 without interfering with hamartin-tuberin complex formation. Moreover, IGF1 and insulin induce tuberin phosphorylation, which is mediated by the PI3K/Akt pathway but not by the MAPK pathway. As a result, cyclin-dependent kinase (CDK) 2 activity, DNA synthesis, and S phase of the cell cycle are elevated. We thus have identified Akt as a major tuberin kinase to negatively regulate hamartin-tuberin tumor suppressor function by inducing degradation.

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Tuberin Is a Physiological Substrate of Akt

Recent studies have demonstrated that because genetic studies of the dTsc complex in Drosophila have demonstrated that dTsc1/DTC2 antagonize insulin signaling in cell growth (10–13), we next examined whether insulin and IGF1 induce hamartin-tuberin phosphorylation and whether Akt mediates this action. Western blotting analyses showed that tuberin, but not hamartin, was phosphorylated upon insulin, IGF1, or serum stimulation in HeLa cells as demonstrated by gel mobility shift (Fig. 1d). The phosphorylation was abrogated by treatment with phosphatase PP2A or PI3K inhibitors, LY294002, and wortmannin, but not by MAPK.

In Vivo (32P)orthophosphate Cell Labeling—COS7 cells were transfected with pcDNA3-TSC2 together with or without constitutively active Akt and labeled with (32P)orthophosphate (0.5 mCi/ml) in minimum Eagle’s medium without phosphate for 4 h. Tuberin was immunoprecipitated with anti-TSC2 antibody. The immunoprecipitates were separated on SDS-PAGE and transferred to membrane. Phosphorylated tuberin was detected by autoradiography.

RESULTS

Tuberin Is a Physiological Substrate of Akt

Akt phosphorylates tuberin in vitro and in vivo and mediates insulin- and IGF1-induced tuberin phosphorylation. a, comparison of the putative Akt phosphorylation sites in tuberin with the sequences of phosphorylation sites of known Akt substrates. The phosphorylated residues are labeled by number, and a consensus sequence is denoted by an arrow (i.e., Ser-Pro-Thr). b, in vitro (32P)orthophosphate labeling HeLa cells transfected with constructs immunoprecipitated with anti-TSC2 antibody are indicated at the top. Immunoprecipitates were separated by SDS-PAGE, transferred to membrane, exposed to the film (upper panel), and detected by anti-TSC2 antibody (bottom panel). WT-Akt, wild type. c, in vitro kinase assay analysis of constitutively active Akt (Myr-Akt) immunoprecipitates using each of the GST-fused seven Akt putative phosphorylation sites as substrate. As shown in Fig. 1c, Akt can highly phosphorylate fusion proteins containing all seven serine and threonine sites of tuberin but not their mutants. We therefore conclude that tuberin is a physiological substrate of Akt.

The PI3K/Akt Pathway, but Not the MAPK Pathway, Mediates Insulin, IGF1, and Serum-induced Tuberin Phosphorylation—Because genetic studies of the dTsc complex in Drosophila have demonstrated that dTsc1/DTC2 antagonize insulin signaling in cell growth (10–13), we next examined whether insulin and IGF1 induce hamartin-tuberin phosphorylation and whether Akt mediates this action. Western blotting analyses showed that tuberin, but not hamartin, was phosphorylated upon insulin, IGF1, or serum stimulation in HeLa cells as demonstrated by gel mobility shift (Fig. 1d). The phosphorylation was abrogated by treatment with phosphatase PP2A or PI3K inhibitors, LY294002, and wortmannin, but not by MAPK.
PI3K/Akt Regulates TSC Tumor Suppressor Complex

Phosphorylation of Tuberin

Because previous studies have suggested that phosphorylation of tuberin regulates its interaction with hamartin (8, 9), we next examined whether Akt interferes with hamartin-tuberin complex formation. Coimmunoprecipitation revealed that expression of wild type and constitutively active Akt in HeLa cells did not disrupt the interaction between hamartin and tuberin (Fig. 2c), despite the fact that hamartin and tuberin function as a complex. Moreover, phosphomimic TSC2-7D-Xpress and nonphosphorylatable TSC2-7A-Xpress, prepared by converting seven Akt phosphorylation sites of tuberin into aspartic acid and alanine, respectively, were transfected into HeLa cells. Immunoblotting analyses of TSC2-7D-Xpress and TSC2-7A-Xpress immunoprecipitates showed that both mutant forms of tuberin still bound to hamartin (Fig. 2d), indicating that Akt phosphorylation of tuberin did not hamper the interaction between hamartin and tuberin.

Hamartin and Tuberin

Strikingly, we observed that expression of constitutively active Akt significantly down-regulated hamartin and tuberin in a dose-dependent manner, i.e. protein levels of hamartin and tuberin progressively declined when the cells were transfected with increasing amounts of constitutively active Akt. Accordingly, the protein amount of hamartin and tuberin in the complex was also decreased (Fig. 3a). To exclude the possibility of Akt down-regulation of hamartin and tuberin resulting from inhibition of the TSC1 and TSC2 gene transcription, Northern blot analyses were performed and showed that mRNA levels of TSC1 and TSC2 did not change in HeLa cells transfected with constitutively active Akt as compared with the cells transfected with pcDNA3 vector alone (Fig. 3e). Because Akt has been shown to activate rather than inhibit translation initiation through regulation of FRAP/mTOR/4E-BP (14, 15), it is unlikely that Akt regulates hamartin and tuberin at a translational level. Thus, we assumed Akt down-regulation of hamartin and tuberin occurred through protein degradation. Pulse-chase experiments revealed that expression of constitutively active Akt considerably induced hamartin and tuberin degradation (Fig. 3b). Moreover, the proteosome inhibitor MG132 attenuated Akt-induced hamartin and tuberin degradation (Fig. 3c). These data suggest that Akt down-regulation of hamartin and tuberin is mediated by a post-transla-

FIG. 2. Akt interacts with tuberin but does not interfere with the hamartin-tuberin complex. Immunoprecipitates prepared from HeLa cells with anti-Akt antibody and detected with monoclonal anti-Myc antibody (top left panel), or vice versa (right). Preimmune serum (Pre. serum) was used as negative control, and total cell lysate (tl) was used as positive control. a, Western blot analyses of TSC2-7D-Xpress and TSC2-7A-Xpress, prepared by converting seven Akt phosphorylation sites of tuberin into aspartic acid and alanine, respectively, were transfected into HeLa cells. Immunoblotting analyses of TSC2-7D-Xpress and TSC2-7A-Xpress immunoprecipitates showed that both mutant forms of tuberin still bound to hamartin (Fig. 2d), indicating that Akt phosphorylation of tuberin did not hamper the interaction between hamartin and tuberin.
FIG. 3. Akt phosphorylation of tuberin induces degradation of hamartin and tuberin. 

a, HeLa cells were transfected with HA-Myr-Akt. A portion of the cell lysate was subjected to Western blot analysis using anti-TSC1 (panel 1), -TSC2 (panel 2), -HA (panel 3), and -β-actin (panel 4) antibodies. The rest were immunoprecipitated with anti-TSC2 antibody and detected with anti-TSC1 antibody (panel 5) or vice versa (panel 6).

b, constitutively active Akt-transfected HeLa cells were pretreated with or without MG132 for 2 h, lysed, and subjected to immunoblotting analyses with anti-TSC1, -TSC2, and -β-actin antibodies.

c, pulse-chase analyses of degradation of hamartin and tuberin. TSC2-positive EEF4 cells were transfected with plasmids indicated at the bottom of each panel, labeled with [35S]methionine, chased at indicated times, and immunoprecipitated with anti-TSC1 or -TSC2 antibodies. The immunoprecipitates were separated by SDS-PAGE, exposed, and quantified.

d, phosphomimic TSC2-7D promotes and nonphosphorylatable TSC2-7A inhibits the degradation of hamartin and tuberin. TSC2-deficient EEF8 cells were transfected with indicated expression plasmids (bottom of each panel) and chased at indicated times after labeling with [35S]methionine. Immunoprecipitations were performed with anti-TSC1, -TSC2, or -Xpress antibodies. Graphical presentations show the normalized density of hamartin and tuberin degradation from 100%.

e, ectopic expression of constitutively active Akt does not affect mRNA levels of TSC1 and TSC2. HeLa cells were transfected with increasing amounts of constitutively active Akt. After 48 h of transfection, total RNAs were isolated and subjected to Northern blot analyses with [32P]dCTP-labeled TSC1 (left) or TSC2 (right) cDNA probe. Bottom panels indicate equal loadings.
tional modification mechanism in which the proteosome pathway is involved.

To examine whether Akt-induced hamartin-tuberin degradation depends upon Akt phosphorylation of tuberin, EEF8 TSC2-deficient cells were transfected with wild type TSC2, phosphomimic TSC2-7D, or nonphosphorylatable TSC2-7A. Western blotting and pulse-chase analyses revealed that TSC2-7D was degraded more rapidly than wild type TSC2, whereas TSC2-7A became more stable. Expression of TSC2-7D promoted hamartin degradation, whereas TSC2-7A stabilized hamartin and inhibited Akt-induced hamartin degradation (Fig. 3d), indicating that Akt phosphorylation of tuberin is required for degradation of the hamartin-tuberin complex. Previous studies have shown that tuberin functions as a cytosolic chaperone protein to prevent hamartin self-aggregation and maintain the tuberin-hamartin complex in a soluble form (9, 19). However, we did not observe that Akt phosphorylation of tuberin affected its chaperone function (data not shown).

**Akt Phosphorylation of Tuberin Leads to Down-regulation of p27<sup>kip1</sup> and Cell Proliferation**

The results from studying altered expression of either TSC1 or TSC2 have demonstrated that both hamartin and tuberin inhibit cell growth and cell size in mammalian (4, 5) and *Drosophila* (10–12), respectively. The mechanism that has been characterized so far is that overexpression of hamartin and tuberin induces the expression of the cyclin-dependent kinase inhibitor p27<sup>kip1</sup> through inhibition of

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**Fig. 4. Akt phosphorylation of tuberin results in p27<sup>kip1</sup> degradation and cell proliferation.**

*Panel a.* Similar to Fig. 4d, HeLa cells were transfected with expression constructs indicated at the bottom of each panel, labeled with [35S]methionine, chased at indicated times, immunoprecipitated with anti-p27<sup>kip1</sup> antibody, and separated by SDS-PAGE. Graphical presentations show the normalized density of p27<sup>kip1</sup> degrading from 100%. *b.* In vitro CDK2 kinase assay analyses of cyclin E-CDK2 immunoprecipitates prepared from cells transfected with indicated plasmids. *c.* In vitro cyclin E-CDK2 kinase assay analyses of cyclin E-CDK2 immunoprecipitates prepared from cells transfected with indicated plasmids. *d.* Cyclin E-CDK2 kinase assay analyses of cyclin E-CDK2 immunoprecipitates prepared from cells transfected with indicated plasmids. *e.* Cyclin E-CDK2 kinase assay analyses of cyclin E-CDK2 immunoprecipitates prepared from cells transfected with indicated plasmids.
its degradation (20, 21). To examine the effects of Akt phosphorylation of tuberin on p27kip1 expression, pulse-chase analyses were performed with TSC2-deficient EEF8 cells that were transfected with TSC2, TSC2/Mry-Akt, TSC2-7D, or TSC2-7A. As shown in Fig. 4a, expression of constitutively active Akt abrogated the ability of stabilization of p27kip1 by tuberin. p27kip1 degraded rapidly in phosphomimic TSC2-7D-transfected cells as compared with the cells expressing wild type TSC2/constitutively active Akt. In contrast, the cells expressing TSC2-7A exhibited similar degradation rate of p27kip1 to wild type TSC2-transfected cells. Moreover, expression of TSC2-7A abrogated constitutively active Akt-induced p27kip1 degradation (Fig. 4a). These data indicate that degradation of p27kip1 is regulated by Akt phosphorylation of tuberin.

Because G1/S CDK2 is a major target of p27kip1 (21), we next examined whether Akt overrides tuberin-inhibited CDK2 activity. Consistent with previous reports (21, 22), expression of wild type TSC2 inhibited CDK2 activity in a dose-dependent manner. However, constitutively active Akt abrogated TSC2-inhibited CDK2 activity (Fig. 4b). Phosphomimic TSC2-7D lost the ability to inhibit CDK2 activity, whereas expression of nonphosphorylatable TSC2-7A displayed the same effects as wild type TSC2 (Fig. 4b).

Because CDK2 is a major regulator of cell growth and G1/S transition of the cell cycle, we further examined the effects of Akt phosphorylation of tuberin on cell proliferation measured by cell growth and thymidine incorporation. As shown in Fig. 4, c and d, expression of TSC2 or TSC2-7A in TSC2-deficient EEF8 cells inhibited cell growth and DNA synthesis as compared with the cells transfected with vector alone. However, cells expressing constitutively active Akt or phosphomimic TSC2-7D significantly enhanced cell growth and thymidine incorporation. Consistent with previous reports (21, 22), the number of cells at the G0–G1 phase of the cell cycle was increased in constitutively active Akt or phosphomimic TSC2-7D-transfected cells (Fig. 4d). These results indicate that Akt phosphorylated tuberin, even though Akt2 displays a slightly higher binding affinity to tuberin, even though Akt2 displays a slightly higher binding affinity to tuberin. The model in Fig. 5 illustrates the mechanism through which the PI3K/Akt pathway mediates insulin and IGF1 signals to down-regulate hamartin-tuberin function by phosphorylation of tuberin. Our results define a possible new mechanism through which Akt induces cell proliferation and transformation by inhibiting TSC1/TSC2 tumor suppressor functions.

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**PI3K/Akt Regulates TSC Tumor Suppressor Complex**

*WITHDRAWN*

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