Cell Cycle-regulated Phosphorylation of Hamartin, the Product of the Tuberous Sclerosis Complex 1 Gene, by Cyclin-dependent Kinase 1/Cyclin B*

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Tuberous sclerosis complex (TSC) is a tumor suppressor gene syndrome whose manifestations can include seizures, mental retardation, and benign tumors of the brain, skin, heart, and kidneys. Hamartin and tuberin, the products of the TSC1 and TSC2 genes, respectively, form a complex and inhibit signaling by the mammalian target of rapamycin. Here, we demonstrate that endogenous hamartin is threonine-phosphorylated during nocodazole-induced G2/M arrest and during the G2/M phase of a normal cell cycle. In vitro assays showed that cyclin-dependent kinase 1 phosphorylates hamartin at three sites, one of which (Thr417) is in the hamartin-tuberin interaction domain. Tuberin interacts with phosphohamartin, and tuberin expression attenuates the phosphorylation of exogenous hamartin. Hamartin with alanine mutations in the three cyclin-dependent kinase 1 phosphorylation sites increases the inhibition of p70S6 kinase by the hamartin-tuberin complex. These findings support a model in which phosphorylation of hamartin regulates the function of the hamartin-tuberin complex during the G2/M phase of the cell cycle.

Tuberous sclerosis complex (TSC) is a tumor suppressor gene syndrome whose manifestations can include seizures, mental retardation, autism, and tumors in the brain, retina, kidney, heart, and skin. Mutations in two genes, TSC1 on chromosome 9q34 (1) and TSC2 on chromosome 16p13 (2), cause TSC. Tuberin, the TSC2 gene product, and hamartin, the TSC1 gene product, are known to interact (3, 4) and appear to function as a complex. Hamartin and tuberin function in multiple cellular pathways in mammalian cells, including vesicular trafficking (5), regulation of the G1 phase of the cell cycle (6–10), steroid hormone regulation (11), and Rho activation (12, 13). Tuberin is phosphorylated by the kinase Akt (protein kinase B) (24, 27–30). Phosphorylation of tuberin by Akt negatively regulates inhibition of p70S6K by tuberin. Tuberin is also a substrate of the p38 and MK2 kinase cascade (31), mediating its interaction with 14-3-3 (32–34). Here, we report that endogenous hamartin is phosphorylated during G2/M, demonstrating for the first time that hamartin, like tuberin, is regulated by phosphorylation. Results from both in vitro and in vivo experiments indicate that hamartin is a substrate of the cyclin-dependent kinase CDK1 (cdc2), which is active in late cell cycle phases and promotes entry into mitosis when bound to cyclin B1 (reviewed in Refs. 35 and 36). Hamartin is phosphorylated by CDK1 at three residues, the most highly conserved of which lies within the hamartin-tuberin interaction domain. A mutant form of hamartin that cannot be phosphorylated by CDK1 increased the inhibition of p70S6K by the hamartin-tuberin complex. These results suggest that regulation of the hamartin-tuberin complex during G2/M may play a role in the integration of cell division with cell size, protein synthesis, and/or growth factor signaling.

EXPERIMENTAL PROCEDURES

Cells and Cell Treatments—HEK293 human embryonic kidney cells (ATCC CRL-1573) were cultured in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum. G2/M arrest was induced by treating the cells for 18.5 h with 70 ng/ml nocodazole or Taxol dissolved in Me2SO (all from Sigma). For cell cycle synchronization, the cells were treated with 500 μM hydroxyurea (Sigma) for 16 h, released in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, and harvested at different time points after release. For CDK1 inhibition, the cells were treated with 20 μM alsterpaullone (A.G. Scientific Inc., San Diego, CA).

Site-directed Mutagenesis—Site-directed mutagenesis was performed using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) and confirmed by DNA sequencing. The constructs were cloned into pcDNA 3.1- or pcDNA3.1- (His) (Invitrogen).

Transfections—The transfections were performed using FuGENE 6 transfection reagent (Roche Applied Science).

Immunoblotting and Antibodies—The cells were lysed in RIPA buffer (1× phosphate-buffered saline, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, supplemented by protease and phosphatase inhibitors, 1% SDS, 20 μM leupeptin) and centrifuged at 15,000 x g for 10 min. The supernatant was used for immunoblotting.
transferred to Immobilon-P membranes (Millipore, Bedford, MA). The following antibodies were used to detect proteins by enhanced chemiluminescence (Amersham Biosciences): anti-tuberin C20 (Santa Cruz, Santa Cruz, CA), anti-hamartin (3), anti-β-actin (Sigma), anti-phosphotyrosine clone 4G10 (Upstate, Waltham, MA), anti-phosphotyrosine clone PY99 (Santa Cruz), anti-phosphothreonine-proline, anti-p70S6K, and anti-phosphoT389-p70S6K (Cell Signaling, Beverly, MA).

**Flow Cytometry Analysis**—The cell pellets were fixed in 70% ethanol and stained with 20 μg/ml propidium iodide (Sigma) containing 9.5 mg/ml RNase (Sigma). Flow cytometry was performed on a Becton-Dickinson FACScan machine. The percentages of cells in the G0, S, and G2/M phases of the cell cycle were determined using CellQuest DNA Acquisition software (Becton-Dickinson).

**Immunoprecipitations**—The cells were lysed on ice in RIPA or PBT buffer (50 mM HEPES, 50 mM NaCl, 5 mM EDTA, 1% Triton X-100, and 50 mM NaF). 500–1000 μg of total protein were incubated at 4 °C with anti-hamartin, anti-tuberin, or anti-TetraHis (Qiagen) antibodies for 1 h with constant rotation. Fifty μl of protein A-Sepharose bead slurry (Invitrogen) were added to the immunocomplexes and rotated at 4 °C for 16 h. The beads were washed in lysis buffer and boiled in Laemmli buffer (Bio-Rad).

**Phosphatase Assays**—The hamartin immunoprecipitates were washed with either CIAP buffer (50 mM Tris-HCl, pH 9.0, 1 mM MgCl2) or PP1 buffer (50 mM Tris-HCl, pH 7.5, 1 mM MnCl2, 1 mM dithiothreitol). The hamartin immunoprecipitates were washed in 500 μl of kinase buffer (40 mM HEPES, 10 mM NaCl, 1 mM MgCl2, 1 mM MnCl2, pH 7) and incubated for 30 min at 30 °C in 25 μl of kinase buffer containing 100 μM ATP and 1 μl of activated CDK1/cyclin B complex. The reaction was terminated by boiling the beads in Laemmli buffer.

**p70S6K in Vitro Kinase Assay**—Activated CDK1/cyclin B complexes were prepared by mixing His-tagged cyclin B bound to nickel beads with baculovirus-expressed recombinant CDK1, in the presence of 50 μM ATP and 10 mM MgCl2 (37). The CDK1/cyclin B complex was eluted from the beads using 62.5 μl of imidazole buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 100 mM imidazole). The hamartin immunoprecipitates were washed in 500 μl of kinase buffer (40 mM HEPES, 10 mM NaCl, 1 mM MgCl2, 1 mM MnCl2, pH 7) and incubated for 30 min at 30 °C in 25 μl of kinase buffer containing 100 μM ATP and 1 μl of activated CDK1/cyclin B complex. The reaction was terminated by boiling the beads in Laemmli buffer.

**p70S6K in Vitro Kinase Assay**—Myc-S6K1 (a gift of Dr. Richard Lamb), TSC1, and TSC2 were transfected in HEK293 cells, and the cell lysates were prepared in PBT buffer after serum deprivation for 24 h. Ectopic p70S6K was immunoprecipitated with Myc-agarose beads (BD Clontech, Palo Alto, CA). The beads were washed in ADBI buffer (20 mM MOPS, pH 7.2, 25 mM β-glycerol phosphate, 5 mM EGTA, 1 mM sodium orthovanadate, 1 mM dithiothreitol), and the p70S6K activity was measured using the S6 kinase assay kit (Upstate). Briefly, the immunocomplexes were incubated at 30 °C for 30 min with a substrate peptide containing the p70S6K consensus phosphorylation site in the presence of PKC/PKA inhibitors, 15 mM MgCl2, 100 μM ATP, and 10 μCi of [γ-32P]ATP. The reactions were spotted on P81 paper, air-dried, and washed in 0.75% phosphoric acid and aceton, and the radioactivity was measured in a scintillation counter.

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**FIG. 1.** Nocodazole-induced G2/M arrest of HEK293 cells results in a second, slower mobility band of endogenous hamartin. A, HEK293 cells were treated with nocodazole for 18.5 h and immunoblotted with anti-hamartin. The cell lysates were separated by 5% SDS/PAGE. An upshift in hamartin mobility (arrowhead), representing an apparent 10-kDa increase in molecular mass, was present after nocodazole treatment. The results are representative of six independent experiments. B, HEK293 cells were treated with nocodazole for 0, 4, 8, 16, 18.5, 24, or 48 h prior to analysis with fluorescence-activated cell sorting and immunoblot. The fluorescence-activated cell sorting profiles and a percentage of cells in the G2/M phase are indicated for each time point. The cell lysates were separated by 5% (top panel) and 4–20% SDS/PAGE (bottom panel). The upshift in hamartin mobility (arrowhead) was evident beginning at 16 h, when the majority of the cells were in the G2/M phase. An actin immunoblot is shown as a loading control.

**FIG. 2.** Endogenous hamartin is threonine-proline-phosphorylated during nocodazole treatment. A, endogenous hamartin was immunoprecipitated (IP) from HEK293 cells after 18.5 h of nocodazole or MeSO control treatment. The immunoprecipitates were incubated for 20 min with CIAP (a serine/threonine/tyrosine phosphatase), PP1 (a threonine/serine phosphatase), or without phosphatase and separated by 7.5% SDS/PAGE. The upshifted hamartin band (arrowhead) was present in the immunoprecipitate with no phosphatase treatment. The upshifted hamartin band was removed after incubation with CIAP or PP1, indicating that during nocodazole treatment hamartin is phosphorylated on threonine and/or serine residues. The results are representative of three independent experiments. B, HEK293 cells were arrested in G2/M after treatment with nocodazole for 18.5 h. The cell lysates and immunoprecipitates were separated by 7.5% SDS/PAGE and immunoblotted. An upshift in hamartin mobility was present after nocodazole in both the lysate and immunoprecipitate (arrowhead). Increased anti-phosphothreonine/proline immunoreactivity was seen in the immunoprecipitated hamartin after nocodazole treatment. The results are representative of three independent experiments. C, in Cos7 cells, an increase in threonine-proline phosphorylation of hamartin was also present after nocodazole treatment. Similar results were seen in a second independent experiment.
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RESULTS

Nocodazole-induced Arrest Results in a Mobility Shift of Endogenous Hamartin—Under normal growth conditions, hamartin migrates predominantly as a single band in a Western immunoblot. During earlier work in which HEK293 cells were arrested with nocodazole (38), a second more slowly migrating band was observed (Fig. 1A). This band, which represented an apparent increase in the molecular mass of hamartin of ~10 kDa, became evident after 16 h of nocodazole treatment when the majority of the cells were in G2/M (Fig. 1B). This upshift in hamartin was also seen in HEK293 cells after Taxol treatment (data not shown).

Endogenous Hamartin Is Threonine-phosphorylated during Nocodazole Arrest—To determine whether the slower mobility band of hamartin was the result of phosphorylation, endogenous hamartin was immunoprecipitated and immunoblotted. As expected, an upshifted band was present in the immunoprecipitate after nocodazole-induced G2/M arrest (Fig. 2A, compare first and second lanes). Treatment of the immunoprecipitate with CIAP for 20 min at 37 °C abolished the upper band, demonstrating that hamartin is phosphorylated after nocodazole treatment. Treatment of the immunoprecipitate with PP1 removed the slower migrating hamartin band (Fig. 2A), suggesting that hamartin is phosphorylated at a serine and/or threonine residue after nocodazole treatment.

Proline-directed kinases such as CDK1 are known to be active in mitotic cells, and the hamartin-tuberin complex has

Fig. 3. Tuberin prevents hamartin phosphorylation and interacts with phosphorylated hamartin. A, HEK293 cells were lysed in PTY buffer. Hamartin was immunoprecipitated from cells with endogenous hamartin expression, ectopic expression of hamartin alone, or co-expression of both hamartin and tuberin. The immunoprecipitates were separated by 5% SDS/PAGE and immunoblotted with anti-hamartin or anti-phosphothreonine-proline antibodies. An increase in phosphothreonine-proline immunoreactivity was seen with nocodazole treatment. Phosphothreonine-proline immunoreactivity was reduced when both hamartin and tuberin were expressed, compared with cells with ectopic expression of hamartin only, suggesting that tuberin co-expression attenuates hamartin threonine-proline phosphorylation. Similar results were seen in three independent experiments. B, HEK293 cells were transfected with His-hamartin and either wild type or a mutant form of tuberin (TSC2R611Q), which does not interact with hamartin. The cells were lysed in PTY buffer after incubation with Me2SO or nocodazole and ectopically expressed hamartin was immunoprecipitated (IP) with anti-His antibody. In the lysates, an upshift in hamartin mobility was observed in the nocodazole-treated TSC1/TSC2 cells (lane 2) and in both untreated and nocodazole-treated TSC1/TSC2R611Q cells (lanes 3 and 4, respectively). Similarly, in the hamartin immunoprecipitates, increased reactivity with the phosphothreonine-proline antibody was detected for the nocodazole-treated TSC1/TSC2 cells and in both Me2SO and nocodazole-treated TSC1/TSC2R611Q cells. C, HEK293 cells with endogenous expression of hamartin and tuberin were treated with Me2SO or nocodazole and lysed in PTY buffer, and either hamartin or tuberin was immunoprecipitated. As expected, a shift in hamartin mobility and increased reactivity to phosphothreonine-proline was observed in the hamartin immunoprecipitates from nocodazole-treated cells. A shift and reactivity to phosphothreonine-proline was also observed in tuberin immunoprecipitates from nocodazole-treated cells, indicating that tuberin can interact with phosphohamartin. D, His-tagged hamartin was co-expressed with tuberin. The cells were treated with nocodazole, and tuberin was co-immunoprecipitated with His-tagged hamartin. The same amount of tuberin co-immunoprecipitated with hamartin from nocodazole-treated or control cells.

Fig. 4. Hamartin is threonine-proline-phosphorylated during G2/M progression in normal cell cycle. A, HEK293 cells were arrested in G1/S with hydroxyurea and harvested at 0, 8, 9, 10, 11, and 12 h after release. Hamartin immunoprecipitates were separated by 7.5% SDS/PAGE, and immunoblotted with anti-hamartin and anti-phosphothreonine-proline antibodies. An increase in threonine-proline phosphorylation was present at 10, 11, and 12 h, coinciding with a high percentage of cells in G2/M. The results are representative of three independent experiments. B, to determine whether hamartin was dephosphorylated as cells progressed past G2/M, the cells were harvested at 0, 8, 10, 12, 16, and 24 h after hydroxyurea release. The immunoprecipitated hamartin was separated by 7.5% SDS/PAGE. The increase in threonine-proline phosphorylation of hamartin was evident 8 h after release when 48% of the cells were in the G2/M phase and then decreased at 16 and 24 h when 27 and 19% of the cells were in the G2/M phase, respectively.
endogenous hamartin is phosphorylated during the normal cell cycle, HEK293 cells were synchronized in the G1 phase with hydroxyurea followed by release into fresh medium. The cells were collected at 0, 8, 9, 10, 11, and 12 h and analyzed by fluorescence-activated cell sorting. Hamartin was immunoprecipitated at each time point and immunoblotted with the hamartin and phosphothreonine-proline antibody (Fig. 4A). An increase in phosphothreonine-proline immunoreactivity was seen, with the highest immunoreactivity 12 h after release when 55% of the cells were in G2/M. An upshift in hamartin mobility was not detected in this experiment. This may indicate that fewer sites are phosphorylated during normal G2/M phase compared with prolonged exposure to active CDK1 during nocodazole-induced arrest. To determine whether the phosphorylation was reversible, the experiment was repeated with later time points (Fig. 4B). The increased phosphorylation was detected at 8, 10, and 12 h after release from hydroxyurea, when 48, 53, and 41% of the cells were in the G2/M phase, respectively, but

Ectopically Expressed Hamartin Is Phosphorylated during Nocodazole Arrest, and Co-expression of Tuberin Attenuates Hamartin Phosphorylation—These experiments demonstrated that endogenous hamartin is threonine-proline-phosphorylated during nocodazole-induced G2/M arrest. To determine whether exogenous hamartin was also threonine-phosphorylated after nocodazole treatment, hamartin was overexpressed in HEK293 cells, immunoprecipitated, and immunoblotted with the phosphothreonine-proline antibody. An increase in threonine-proline-phosphorylated hamartin was observed in nocodazole-treated cells (Fig. 2B), indicating that hamartin is phosphorylated at a threonine-proline residue and consistent with the result of the PP1 phosphatase experiment (Fig. 2A). In contrast, no immunoreactivity was observed with either of two phosphotyrosine-specific antibodies after nocodazole treatment (data not shown). The increase in threonine-proline phosphorylation of hamartin after nocodazole treatment was also seen in Cos7 cells (Fig. 2C). Nocodazole did not induce threonine-proline phosphorylation of tuberin (data not shown).

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Hamartin is phosphorylated when complexed with tuberin.

nocodazole (Fig. 3) (40), is phosphorylated even in the absence of nocodazole (Fig. 3B). This suggests that hamartin may be protected from phosphorylation when complexed with tuberin.

Phosphorylation of Hamartin Does Not Alter the Interaction with Tuberin—An interaction between endogenous tuberin and endogenous phosphorylated hamartin was detected in tuberin immunoprecipitates of nocodazole-treated cells (Fig. 3C). No difference in the amount of co-immunoprecipitated tuberin was observed in His immunoprecipitates from cells expressing TSC2 and His-TSC1 (Fig. 3D). Taken together, these data indicate that phosphorylation of hamartin does not have a major effect on the interaction between hamartin and tuberin.

Endogenous Hamartin Is Phosphorylated during the G2/M Phase of the Cell Cycle—Phosphorylation during nocodazole-induced arrest could indicate regulation of hamartin during G2/M phases of the normal cell cycle or alternatively reflect the cell response to microtubular damage. To determine whether
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not at 16 h, when only 27% of the cells were in the G2/M phase. A small amount of baseline phosphothreonine proline immunoreactivity was present at the 0- and 24-h time points, which could indicate persistence of the phosphorylation by CDK1 or the activity of another kinase.

Hamartin Is Phosphorylated in Vivo by CDK1/Cyclin B—To determine whether CDK1 phosphorylates hamartin in vivo, immunoprecipitated hamartin was incubated with activated CDK1/cyclin B complexes. The amount of total protein used was normalized to the extent of overexpression prior to hamartin immunoprecipitation. An upshift in the mobility of endogenous hamartin was present after incubation with CDK1/cyclin B (Fig. 5A). A similar upshift was seen after kinase treatment of hamartin immunoprecipitates with cells expressing ectopic hamartin only or co-expression of hamartin and tuberin (Fig. 5A). To establish the specificity of the CDK1/cyclin B complex on hamartin, the hamartin immunoprecipitates were incubated with two kinase-dead forms of CDK1 (T161A and N133A). The mobility shift was not observed with either kinase-dead form of CDK1 (Fig. 5B). To verify that the change in the mobility of hamartin was the result of phosphorylation, the product of the kinase assay was treated with CIAP or the kinase-dead form of CDK1 (Fig. 5C). The preferred consensus phosphorylation sequence for CDK1 is (S/T)P (41). Hamartin contains three potential CDK1 sites: Thr417, Ser584, and Thr1047 (Fig. 7A). Thr417, the most highly evolutionarily conserved of the three potential sites, is within the hamartin-tubulin interaction domain (residues 302–312) (12). These sites were mutated to alanine either singly (TSC1–417A–1047A), doubly (TSC1–417A–1047A–1047A), or all three (TSC1AAA). These forms of hamartin were overexpressed with wild-type TSC2, immunoprecipitated, and tested for in vitro phosphorylation by CDK1. As shown in Fig. 7B, both of the single mutants showed an upshift in hamartin after incubation with CDK1/cyclin B, although the degree of the shift was less than that of wild-type hamartin. This result indicated that neither Thr417 nor Thr1047 was the sole site of in vitro CDK1 phosphorylation. The double mutant also showed a shift. The

Fig. 7. Hamartin contains three consensus CDK1 phosphorylation sites ((S/T)PX(K/R)). A, the evolutionary conservation of the three consensus CDK1 phosphorylation sites of hamartin is shown for human, rat, and Drosophila melanogaster. Of the three sites (Thr417, Ser584, and Thr1047), only Thr417 is conserved in all three species. Thr417 is within the tubulin interaction domain of hamartin, and Thr1047 is within the ezrin-radixin-moesin (ERM) interaction domain, as illustrated. B, to determine which potential CDK1 sites were phosphorylated, the in vitro kinase assay was performed with immunoprecipitated (IP) hamartin from cells expressing wild-type hamartin (wt-TSC1) or one of the following hamartin phosphomutants: T417A (TSC1–417A), T1047A (TSC1–1047A), T417A/T1047A double phosphomutant (TSC1–417A/1047A), and T417A/S584A/T1047A triple phosphomutant (TSC1AAA). The hamartin upshift (arrowhead) was evident after 5% SDS/PAGE for all forms of hamartin except the triple phosphomutant. The relative degree of upshift was less for each single and for the double phosphomutant compared with wild-type hamartin.
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FIG. 8. The hamartin triple phosphomutant is not threonine-proline-phosphorylated during nocodazole-induced G_{2}/M arrest or during normal G_{2}/M progression. A, His-tagged wild-type (His-TSC1) or triple phosphomutant hamartin (His-TSC1AAA) was co-expressed with tuberin in HEK293 cells, immunoprecipitated (IP) using anti-His antibody, separated by 7.5% SDS/PAGE, and immunoblotted with anti-hamartin or anti-phosphothreonine-proline antibodies. The hamartin triple phosphomutant was not threonine-proline-phosphorylated during nocodazole treatment, consistent with the in vitro results in Fig. 7. Similar results were seen in three independent experiments. B, HEK293 cells were transfected with His-TSC1/TSC2 or His-TSC1AAA/TSC2, arrested in G_{1}/S with hydroxyurea, and harvested at 8, 10, and 12 h after release. The cells were lysed in RIPA buffer and electrophoretically expressed hamartin was immunoprecipitated with anti-His antibody. The immunoprecipitated wild-type hamartin showed increased reactivity for the phosphothreonine-proline antibody at 10 and 12 h (similar to Fig. 4). This increase in immunoreactivity was not detected with the phosphomutant form of hamartin (TSC1AAA).

The hamartin phosphorylation during nocodazole-induced G_{2}/M arrest or during normal G_{2}/M progression was modified during nocodazole treatment. The fact that endogenous hamartin is phosphorylated during the normal cell cycle suggests that hamartin phosphorylation has physiologic significance. The most highly conserved consensus CDK1 phosphorylation site of hamartin lies within the hamartin-tuberin interaction domain. However, we did not detect a difference in the interaction between hamartin and tuberin during nocodazole arrest. We also did not detect a difference in the cell cycle profile of asynchronous HEK293 cells or their ability to arrest in nocodazole when tuberin was co-expressed with the three forms of hamartin (data not shown).

Expression of the Triple Phosphomutant Hamartin Does Not Block Nocodazole-induced Arrest and Does Not Alter the Cell Cycle Profile of HEK293 Cells—To determine whether phosphorylation of hamartin inhibits the ability of cells to arrest in nocodazole or alters cell cycle kinetics, tuberin was overexpressed with wild-type hamartin or triple phosphomutant hamartin (TSC1AAA). No differences were observed in the cell cycle profile of asynchronous or nocodazole-arrested cells (data not shown).

Phosphomutant Hamartin Increases the Inhibition of p70S6K—To study the effect of hamartin phosphorylation on the activation of the mTOR/p70S6K pathway, HEK293 cells were co-transfected with Myc-S6K1 together with wild-type TSC2 and either wild-type TSC1 or TSC1AAA. Following immunoprecipitation of Myc-p70S6K, the activity of p70S6K was measured by an in vitro kinase assay. As previously reported by several groups, the p70S6K activity was decreased by the expression of the hamartin-tuberin complex. Expression of the nonphosphorylatable TSC1AAA further decreased the activity of p70S6K, compared with TSC1/TSC2 transfected cells (Fig. 9A). Western immunoblotting confirmed that the phosphorylation of p70S6K at residue Thr^{389} was decreased in cells transfected with hamartin phosphomutant compared with wild type both in the total cell lysates and in p70S6K immunoprecipitates (Fig. 9B). We then tested the tuberin AKT phosphomutant S939A/T1482A (TSC2AA) (27). The magnitude of suppression of p70S6K phosphorylation was similar when TSC1AAA was expressed with wild-type TSC2, compared with wild-type TSC1 with TSC2AA. Co-expression of TSC1AAA with TSC2AA further decreased the phosphorylation of p70S6K (Fig. 9C).

DISCUSSION

We report here that endogenous hamartin is threonine-phosphorylated during nocodazole-induced G_{2}/M phase and also during the G_{2}/M phase of the normal cell cycle. Both in vitro and in vivo studies indicate that hamartin is a direct target for phosphorylation by CDK1. In vitro, hamartin is phosphorylated at three sites (Thr^{417}, Ser^{584}, and Thr^{1047}) by CDK1. In vivo, hamartin phosphorylation is blocked by the CDK1 inhibitor alsterpaullone, and hamartin with the three residues mutated to alanine (TSC1AAA) is not threonine-proline-phosphorylated during nocodazole-induced G_{2}/M arrest or during G_{2}/M progression of the normal cell cycle. Together, these findings indicate that CDK1 is the kinase responsible for the phosphorylation. Another group has previously reported that the hamartin-tuberin complex co-immunoprecipitates with CDK1, cyclin B1, and cyclin A (39), supporting the hypothesis that hamartin is directly phosphorylated by CDK1. To our knowledge, this is the first indication that endogenous hamartin is phosphorylated and that the hamartin-tuberin complex is specifically regulated during the cell cycle.

The fact that endogenous hamartin is phosphorylated during the normal cell cycle suggests that hamartin phosphorylation has physiologic significance. The most highly conserved consensus CDK1 phosphorylation site of hamartin lies within the hamartin-tuberin interaction domain. However, we did not detect a difference in the interaction between hamartin and tuberin during nocodazole arrest. We also did not detect a difference in the cell cycle profile of asynchronous HEK293 cells or their ability to arrest in nocodazole when tuberin was co-expressed with the three forms of hamartin (data not shown).

Phosphorylated hamartin from cells with endogenous expression of the two proteins co-immunoprecipitates with tuberin, and the amount of tuberin co-immunoprecipitating with hamartin is similar in nocodazole-treated and untreated cells. Co-expression of both tuberin and hamartin reduced the phosphorylation of hamartin both in vitro and in vivo, compared with expression of hamartin alone. Hamartin is constitutively phosphorylated when co-expressed with a patient-derived TSC2 mutant, which has been previously shown not to interact with hamartin (40). These data suggest that tuberin attenuates hamartin phosphorylation and/or that the primary substrate for phosphorylation is hamartin not complexed with tuberin.

Studies from both Drosophila and mammalian cells have demonstrated that the hamartin-tuberin complex negatively regulates mTOR and p70S6K (9, 19, 23–25). During mitosis, the activity of p70S6K is suppressed, in part by direct phosphorylation of the p70S6K autoinhibitory domain by CDK1 (44,
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45). It is known that cell cycle progression and cell growth are coordinated, yet separable, in mammalian cells (43) as well as in yeast and Drosophila, but the signaling pathways responsible for this coordination are not yet completely understood.

We hypothesize that phosphorylation of hamartin by CDK1 in the G2/M phase regulates the activity, rather than the interaction, of the hamartin-tuberin complex during mitosis. If this is true, the phosphorylation of hamartin by CDK1 could play a central role in the coordination of cell cycle progression, cell growth, and cell size. Supporting this hypothesis, we found that a nonphosphorylatable form of hamartin (TSC1AAA) inactivates p70S6K during early mitosis. During late mitosis/early G1 phase, the phosphorylation of hamartin by CDK1 inactivates p70S6K during early mitosis. During late mitosis/early G1 phase, the phosphorylation of hamartin by CDK1 could release the inhibition on p70S6K, allowing for increased protein synthesis and cellular growth. Further studies will be needed to address these issues.

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