Tuberin Binds p27 and Negatively Regulates Its Interaction with the SCF Component Skp2*

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TSC1 (tuberous sclerosis complex 1) encoding hamartin and TSC2 encoding tuberin are tumor suppressor genes responsible for the autosomal dominantly inherited disease tuberous sclerosis. These genes have been demonstrated to negatively regulate cell cycle progression, the activity of cdk2, and the degradation of the cyclin-dependent kinase inhibitor p27. To date, the underlying molecular mechanism remains elusive. Here, we show that tuberin binds to p27. Whereas tuberin also binds p27 in TSC1-negative cells, hamartin does not bind p27 without tuberin. p27 protein levels are regulated through ubiquitin-dependent degradation. Skp2 is the F-box protein, which, together with other proteins, forms an SCF (Skp1/cullin/F-box protein)-type E3 ubiquitin ligase complex whose task is to target p27 for degradation by the proteasome. We found that neither tuberin nor hamartin are in a complex with Skp2. Tuberin does not affect Skp2 protein levels, and the SCF$^{\text{Skp2}}$ ubiquitin ligase does not regulate tuberin stability. But binding of tuberin to p27 sequesters p27 from Skp2 and triggers an up-regulation of the amount of p27 stability.

Tuberous sclerosis complex (TSC)† is an autosomal dominant disease occurring in ~1 in 6000 live births. It is characterized by the development of benign tumors called hamartomas in the kidneys, heart, skin, and brain. The latter often cause seizures, mental retardation, and a variety of developmental disorders, including autism (1). TSC1 on chromosome 9q34 encodes hamartin (2) and TSC2 on chromosome 16p13.3 encodes tuberin (2). TSC patients carry a mutant TSC1 or TSC2 gene in each of their somatic cells, and loss of heterozygosity has been documented in a wide variety of TSC tumors. In mammalian cells hamartin and tuberin form a complex, providing a tentative explanation for the similar disease phenotype in TSC patients with mutations in either of these genes (4, 5).

The TSC proteins have been implicated in the regulation of different cellular functions such as endocytosis, transcription, neuronal differentiation, or cell adhesion (1). Recent studies of Drosophila and mammalian cells demonstrated that the TSC1-TSC2 complex functions as a GTPase-activating protein against Rheb (Ras homolog enriched in brain), which, in turn, regulates mTOR (mammalian target of rapamycin) signaling. Several investigations have shown that the TSC1-TSC2 complex antagonizes the mTOR signaling network, which has a central role in the regulation of cell growth in response to growth factors, cellular energy, and nutrient levels (recently reviewed in Refs. 6 and 7).

Work on mammalian cells has implicated the TSC proteins in cell cycle regulation. In the mammalian cell cycle, the transition from the G0/G1 phase to S phase, in which DNA replication occurs, has been shown to be regulated by cyclin-dependent kinases (cdks). Mitogen-dependent Dtype cyclins are expressed first during early G1. These cyclins associate with cdk4 or cdk6 to form an active kinase complex that phosphorylates the retinoblastoma protein and thereby activates E2F transcriptional activity. Among the genes activated via members of the E2F protein family are cdk E, which, complexed with cdk2, promotes the G1/S transition by phosphorylation of different substrates, and cdk A, which activates cdk2 to further initiate DNA replication (8). In addition, two families of cdk inhibitors are known, namely the INK4 family (p15, p16, p18, and p19), which regulates cdk4 and cdk6, and the Cip/Kip family (p21, p27, and p57), which inhibits a broader range of cdk (9). Antisense inhibition of TSC2 expression induces quiescent fibroblasts to enter the cell cycle, and TSC2-negative cells exhibit a shortened G1 phase. Overexpression of TSC1 or TSC2 negatively regulates cell cycle progression. Tuberin down-regulates the activity of cdk2, and p27 stability is decreased in tuberin-negative cells (10–12). Whereas p27 mRNA expression is not regulated during the cell cycle, degradation of p27 protein by ubiquitin-dependent proteolysis is a critical event for cell cycle progression (13). The attachment of a chain of ubiquitin molecules to a protein by the concerted efforts of E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme), and E3 (ubiquitin ligase) targets the substrate for degradation by the 26 S proteasome (14, 15). Skp2 is the F-box protein, which, together with Skp1, Cul-1, and Roc1/Rbx1, forms an SCF (Skp1/cullin/F-box protein)-type E3 ubiquitin ligase complex whose task is to target p27 for degradation by the proteasome (16–18).

Here, we show that tuberin binds p27. Tuberin sequesters p27 from Skp2 and triggers an up-regulation of the amount of p27 bound to cdk2. Tuberin abolishes Skp2-mediated p27 degradation and cell cycle progression. This finding suggests how p27 levels can be down-regulated in tuberous sclerosis hamartomas without mutation or loss at the p27 gene. Regulation of

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† The abbreviations used are: TSC, tuberous sclerosis complex; cdk, cyclin-dependent kinase; E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating protein; E3, ubiquitin ligase; MEF, mouse embryonic fibroblast.

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FIG. 1. Tuberin interacts with p27 in different cell lines. A, 500-μg protein extracts of logarithmically growing HEK 293 cells, EEF4 cells, and HeLa cells were used to perform immunoprecipitations (IP) with an anti-p27 antibody (p27 C-19; Santa Cruz Biotechnology) (lanes 3, 7, and 11). After SDS-PAGE, the precipitates were immunoblotted with an anti-tuberin antibody (tuberin C-20; Santa Cruz Biotechnology) and an anti-p27 antibody (p27 C-19; Santa Cruz Biotechnology). Immunoprecipitations without antibody were co-analyzed as negative controls (lanes 4, 8, and 12). In addition, 50-μg total lysates were loaded as controls (lanes 1, 5, and 9). To avoid overlapping signals, lanes 2, 6, and 10 have not been loaded. B, logarithmically growing HEK 293 cells were transfected with mammalian expression vectors, either empty (as negative control) or harboring full-length human TSC2 cDNA or FLAG-tagged full-length p27 cDNA. 300-μg protein extracts were used for immunoprecipitations (IP) with an anti-FLAG antibody (M2; Sigma) (lanes 1-3), and the precipitates were immunoblotted with anti-tuberin (tuberin C-20; Santa Cruz Biotechnology), anti-FLAG (M2; Sigma), and an anti-p27 antibody (p27 C-19; Santa Cruz Biotechnology). In addition, 50-μg total lysates of these experiments were analyzed by immunoblot detection with an anti-tuberin antibody (tuberin C-20; Santa Cruz Biotechnology) to confirm TSC2 overexpression (shown at bottom). C, HEK 293 cells were transfected with mammalian expression vectors, either empty or harboring human TSC2 cDNA or mouse p27 cDNA. Immunoprecipitations (IP) with an anti-tuberin antibody (tuberin C-20; Santa Cruz Biotechnology) (lanes 1-3) or anti-p27 antibody (p27 C-19; Santa Cruz Biotechnology) (lanes 4-6) were performed, and the precipitates were immunoblotted with anti-p27 (p27 C-19; Santa Cruz Biotechnology) and an anti-tuberin antibody (tuberin C-20; Santa Cruz Biotechnology). D, 500-μg extracts of logarithmically growing p27-negative and p27-positive MEFs were used to perform immunoprecipitations (IP) with an anti-p27 antibody (p27 C-19; Santa Cruz Biotechnology) (lanes 3 and 4). The precipitates were immunoblotted with anti-p27 (p27 C-19; Santa Cruz Biotechnology) and an anti-tuberin antibody (tuberin C-20; Santa Cruz Biotechnology). In addition, 50-μg total lysates were loaded as controls (lanes 1 and 2).
the protein-ubiquitin ligase and activation of the substrate are known strategies to target cell cycle regulatory proteins for ubiquitination. Here, we demonstrate that substrate protection via binding to a tumor suppressor protein is an additional mechanism.

**EXPERIMENTAL PROCEDURES**

**Cells and Tissue Culture**—HEK (human embryonic kidney) 293 cells and HeLa (human cervical carcinoma) cells were obtained from the American Type Culture Collection, (Manassas, VA). EEF4 cells are rat embryonic fibroblasts (11), MEF p27+/− and MEF p27−/− cells are p27-positive and p27-negative mouse embryonic fibroblasts (19), CACL-1-II cells are TSC1-negative mouse renal carcinoma cells (20), and LdkR cells are TSC2-negative rat renal carcinoma cells (21). All cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) or DMEM/F12 (1:1) supplemented with 10% fetal calf serum and antibiotics (30 mg/ml penicillin and 50 mg/liter streptomycin sulfate). All cultures were kept at 37 °C and 7% CO2 and routinely screened for mycoplasma.

**Transfections**—For transfections, 1 μg per 60-mm Petri dish of the following plasmids was used: the empty pcDNA3 vector; pcDNA3 harboring full-length human wild-type TSC2; pcDNA3 harboring full-length mouse p27; the empty pFLAG-CMV2 vector; FLAG-tagged wild-type human p27 in pFLAG-CMV2; FLAG-tagged T187A p27 mutant (22); FLAG-tagged human wild-type Skp2 in pcDNA3; and FLAG-tagged F-box-deleted mutant Skp2ΔF (16). Cell transfections were performed using the LipofectAMINE reagents obtained from Invitrogen (22); FLAG-tagged human wild-type Skp2 in pcDNA3, and FLAG-tagged F-box-deleted mutant Skp2ΔF (16). Cell transfections were performed using the LipofectAMINE reagents obtained from Invitrogen following the transfection protocol provided by the manufacturer.

**Immunoblotting**—Protein extracts were prepared in buffer containing 20 mM Hepes, pH 7.9, 0.4 mM NaCl, 2.5 mM KCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM NaF, 0.5 mM Na3VO4, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 0.3 μg/ml benzamidin chloride, 10 μg/ml trypsin inhibitor, and 0.5 μg dithiothreitol. Cells were lysed by freezing and thawing. After 20 min on ice, the extracts were centrifuged, and the supernatants were stored at −70 °C. Protein concentrations were determined using the Bio-Rad protein assay reagent with bovine serum albumin as the standard. Proteins were run on an SDS-polyacrylamide gel and transferred to nitrocellulose. Blots were stained with Ponceau S to visualize the loaded protein (23). Immunodetection was performed using another antibody. HEK 293 cells were transfected with FLAG-tagged p27. In protein extracts of these transfected cells, co-immunoprecipitation of tuberin with an anti-FLAG antibody (p27 C-19; Santa Cruz Biotechnology), anti-p27 antibody (p27 T-19; Santa Cruz Biotechnology), anti-FLAG antibody (M2, Sigma), or anti-Skp2 antibodies (N-19; Santa Cruz Biotechnology or anti-Skp2 CL8D9, Zymed Laboratories Inc.). After complex formation, immunoprecipitates were washed twice with buffer containing 1% Nonidet P-40, 150 mM NaCl, 50 mM Tris-Cl, pH 8, 10 mM β-glycerophosphate, 1 mM NaF, 0.1 mM Na3VO4, 0.2 mM phenylmethylsulfonyl fluoride, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 0.3 μg/ml benzamidin chloride, and 10 μg/ml trypsin inhibitor. Western blot analysis and the antibodies used for immunoblot detection are described above. The antibodies used are noted in the legends to Figs. 1–6.

**Cell Cycle Progression Assays**—To study the effects of gene overexpression on cell cycle progression, the empty cytomegalovirus expression vector pcDNA3, FLAG-tagged full-length human wild-type Skp2 in pcDNA3, and the Skp2-pcDNA3 vector together with pcDNA3 harboring wild-type human TSC2 were transfected into mouse embryonic fibroblasts using the LipofectAMINE reagents (described above). Selection for transfected cells was started 24 h after transfection. During the first 2 days of selection the G418 concentration was 1000 μg/ml medium; thereafter the G418 concentration was set at 2000 μg/ml. Starting with equal cell numbers (harvesting and reseeding), the cell pools were grown under G418 selection for 7 days, and cell numbers were determined on the CASY cell counter and analyzer. For these analyses, cells growing in Petri dishes were washed with buffer and harvested by trypsinization (12, 23, 24).

**Statistical Analysis**—Cell cycle progression assay data are given in percentage relative to the mean value of all controls set as 100%. The cell numbers are presented as the mean ± S.D. The significance of the observed differences was determined by Student’s t test (paired; two-tailed) using GraphPad INSTAT software. p values >0.05 are defined as not significant.

**RESULTS**

**Tuberin Binds to p27**—Tuberin is known to be a potent regulator of p27 stability (11). To further elucidate the underlying molecular mechanism, we performed immunoprecipitations using protein extracts of logarithmically growing HEK 293 cells, EEF4 cells, and HeLa cells with a p27-specific antibody. Immunoblot analyses revealed that tuberin can be detected in p27-containing protein complexes in all three cell lines (Fig. 1A). To confirm this interaction of p27 and tuberin by using another antibody, HEK 293 cells were transfected with FLAG-tagged p27. In protein extracts of these transfected cells, co-immunoprecipitation of tuberin with an anti-FLAG antibody could be detected (Fig. 1B). The anti-FLAG antibody leads to a slightly disproportionate signal (compare the signals specific for FLAG and for p27 in Fig. 1B), which is likely to be due to the high specificity of the anti-FLAG antibody. In the analyzed logarithmically growing cell lines, endogenous p27 levels have been found to be low, as is expected in cycling cells (Fig. 1A, p27 short exposure and p27 long exposure). Accordingly, p27 can only be detected in tuberin-specific immunoprecipitations after the overexpression of p27 (Fig. 1C). The find-
ing that transfection of ectopic p27 is required to visualize p27 in tuberin-specific immunoprecipitates in agreement with previous results demonstrating that p27 could not be detected in a tuberin immunoprecipitation assay in untransfected cells (11). To provide further confirmation that the detection of tuberin in immunoprecipitates with the anti-p27 antibody specifically depends on p27 interacting with tuberin, we used protein extracts of p27-negative cells. We found that the anti-p27 antibody does not co-immunoprecipitate tuberin in p27-negative cells (Fig. 1D). Collectively, these data demonstrate a specific physical interaction of tuberin and p27 in vivo in four different cell lines of three species (human HEK 293 cells, human HeLa cells, rat EEF4 cells, and mouse embryonic fibroblasts).

Hamartin Is in the Tuberin-p27 Complex—Hamartin and tuberin form a heterodimer, and this interaction appears to be important for the stability of both proteins (4–6). Therefore, hamartin and tuberin are usually considered to be a complex with one function. p27-specific immunoprecipitations followed by immunoblot detection of tuberin, hamartin, and p27 revealed that hamartin can also be found in a complex with p27 in logarithmically growing HEK 293 cells and HeLa cells (Fig. 2). To investigate the role of the interaction of tuberin and hamartin for the complex formation with p27, we used TSC1-negative CACL-1-II cells and TSC2-negative Lk9dR cells. Tuberin could be detected in p27-specific immunoprecipitates of hamartin-negative CACL-1-II cells (Fig. 2). This finding demonstrates that the binding of tuberin to p27 does not depend on tuberin’s interaction with hamartin. On the contrary, hamartin cannot be detected in a complex with p27 in tuberin-negative Lk9dR cells (Fig. 2). These data suggest that the effects of tuberin on p27 expression could be due to direct interaction, whereas hamartin affects p27 by its influences on tuberin stability/activity. This idea also fits with the earlier observation that the effects of hamartin on p27 are less pronounced than the effects of tuberin (11, 12, 24).2 Interestingly, earlier findings suggested that hamartin can also regulate cell proliferation in tuberin-negative cells, but without effects on p27 protein levels (24). Although the molecular mechanism for this additional capacity of hamartin remains elusive, the finding that hamartin is not in a complex with p27 in tuberin-negative cells could explain why hamartin cannot regulate p27 in these cells.

Tuberin and Hamartin Are Not in a Complex with Skp2—Skp2 is the F-box protein, which is part of an E3 ubiquitin ligase complex whose task is to target p27 for degradation by the proteasome. Skp2 is known to interact with cyclin A, cdk2, and p27, but not with cyclin D1 (16, 25). Skp2-specific immunoprecipitation using extracts of HEK 293 cells followed by immunoblot detection confirmed the interaction of Skp2 with cyclin A, cdk2, and p27 (Fig. 3A). Because Skp2 and tuberin share two capacities, i.e. both bind to p27 and both are involved in the regulation of p27 stability, we wondered whether Skp2 and tuberin can be found in a complex. Under the described experimental conditions, neither tuberin nor hamartin could be detected in Skp2-specific immunoprecipitates (Fig. 3A). These data provide evidence that two p27 complexes exist in the cell, i.e. p27 associated with tuberin and hamartin (without Skp2) and p27 associated with Skp2 (without tuberin and hamartin). Phosphorylation of p27 on Thr-187 has been shown to play a role for its Skp2-dependent degradation (25). To investigate the role of this phosphorylation site for the binding of p27 to tuberin, we ectopically overexpressed FLAG-tagged wild-type p27 and a FLAG-tagged T187A p27 mutant in HEK 293 cells. Immunoprecipitations with an anti-FLAG antibody followed by

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2 M. Rosner and M. Hengstschläger, unpublished observation.
Tuberin Regulates p27-Skp2 Interaction

A

HEK 293

control

TSC2

lysate

Tuberin

Skp2

p27

cdk2

α-tubulin

IP: p27

Tuberin

cdk2

p27

p-cdk2

B

HEK 293

control

IP: Skp2

IP: Skp2

IP: Skp2

IP: Skp2

IP: Skp2

IP: Skp2

TSC2

1 2 3 4 5 6

IgG L

p27

Skp2

Fig. 4. Tuberin regulates p27 expression, p27-cdk2 complex formation, and p27-Skp2 complex formation. A, HEK 293 cells were transfected with mammalian expression vectors, either empty as a control (lane 1) or harboring full-length human TSC2 cDNA (lane 2). 50-µg total protein lysates were used to perform Western blot analysis with antibodies specific for tuberin (tuberin C-20; Santa Cruz Biotechnology), Skp2 (Skp2 H-435; Santa Cruz Biotechnology), p27 (p27 C-19; Santa Cruz Biotechnology), cdk2 (M-2; Santa Cruz Biotechnology), and α-tubulin (Ab-1; Oncogene) (top). In this analysis, cdk2 and phosphorylated cdk2 (p-cdk2) are not separated because of lower gel concentration. In addition, 500-µg protein extracts of these experiments were used to perform immunoprecipitations (IP) with an anti-p27 antibody (p27 C-19; Santa Cruz Biotechnology) followed by immunoblotting using anti-tuberin (tuberin C-20; Santa Cruz Biotechnology), anti-cdk2 (M-2; Santa Cruz Biotechnology), and an anti-p27 antibody (p27 C-19; Santa Cruz Biotechnology) (bottom). B, 1.5 mg protein extracts of cells transfected with the empty vector as control (lane 3) or the expression vector harboring TSC2 (lane 4) were used to perform immunoprecipitations (IP) with an anti-Skp2 antibody (anti-Skp2 CL8D9; Zymed Laboratories Inc.) (lane 3 and 4) and immunoblotting with an anti-p27 (p27 C-19; Santa Cruz Biotechnology) and an anti-Skp2 antibody (Skp2 H-435; Santa Cruz Biotechnology). A 1.5-mg protein extract was used for immunoprecipitation with control IgG as a negative control (lane 5). A 500-µg protein extract was used for immunoprecipitation with an immunodetection revealed that tuberin binds both the wild-type p27 and the T187A p27 mutant with very similar efficiency (Fig. 3B). These data suggest that this phosphorylation is not involved in the control of the tuberin-p27 complex formation.

Tuberin Controls p27 Expression and Its Binding to cdk2 and Skp2—To investigate the effects of tuberin on the expression and binding capacities of Skp2, p27, and cdk2, we transfected logarithmically growing HEK 293 cells with TSC2. Whereas the well known up-regulation of p27 protein levels upon TSC2 overexpression could be confirmed, high levels of tuberin did not affect the endogenous protein expression of Skp2 or cdk2 (Fig. 4A, top). Immunoprecipitation with an anti-p27 antibody followed by immunodetection demonstrated that high ectopic levels of tuberin trigger an up-regulation of tuberin bound to p27 and p27 bound to cdk2 (Fig. 4A, bottom). Under the same experimental conditions, the amount of Skp2 bound to p27 remained unaffected by ectopic tuberin (Fig. 4B). This provides evidence for a molecular model of how tuberin triggers up-regulation of endogenous p27 levels. Knowing that tuberin does not interact with Skp2, the binding of tuberin might protect p27 from binding to Skp2 and from degradation via the proteasome. High levels of ectopic tuberin further induce the interaction of p27 with cdk2. This provides an explanation for the earlier observed regulation of cdk2 activity and cell cycle progression by tuberin (10, 11).

Tuberin Abolishes Skp2-mediated Down-regulation of p27—Next, we wanted to investigate the effects of tuberin on the Skp2-mediated control of p27 protein levels. The potency of ectopic Skp2 to down-regulate endogenous p27 levels is known to be high in G(0)-arrested cells but lower in logarithmically growing cells (17). We found ectopic wild-type Skp2 to be able to negatively affect p27 levels in logarithmically growing HEK 293 cells (Fig. 5, A and B). To prove that this finding is due to Skp2 fulfilling its function as an F-box protein in a SCF-type E3 ubiquitin ligase complex, we demonstrated that an ectopically overexpressed F-box-deleted Skp2 mutant cannot regulate p27 under these conditions (Fig. 5A). Another prerequisite for our model is that Skp2 should not be able to mediate degradation of tuberin. Indeed, in the experiments described above, ectopic Skp2 has no effect on endogenous tuberin protein levels (Fig. 5, A and B). The comparison of cells overexpressing Skp2 alone with cells co-overexpressing Skp2 together with TSC2 revealed that tuberin has the capacity to abolish the Skp2-mediated down-regulation of p27 protein levels (Fig. 5B). These data provide evidence that tuberin can protect p27 from the activity of Skp2 to induce its degradation via the proteasome.

Tuberin Sequesters p27 from Skp2 and Up-regulates p27 Bound to cdk2 in Skp2 Overexpressing Cells—The complex of Skp2 and p27 does not seem to be highly abundant in the cell system used here (compare Fig. 3A with Fig. 4B). A possible explanation for our observations that high levels of ectopic tuberin bind and stabilize p27 and that the amount of p27 bound to Skp2 remains equal could be that the endogenous Skp2 level is rate-limiting under the chosen experimental conditions. We next wanted to test whether tuberin can also actively interfere with the complex formation between Skp2 and p27 in cells expressing high Skp2 levels. HEK 293 cells were transfected with Skp2 alone or were co-transfected with Skp2 together with TSC2. Immunoprecipitations with an anti-p27
FIG. 5. Tuberin sequesters p27 from Skp2 and abolishes Skp2-mediated p27 degradation. A, HEK 293 cells were transfected with mammalian expression vectors, either empty as a control (lane 3) or harboring wild-type (wt) human Skp2 cDNA (lane 2) or an F-box-deleted Skp2ΔF mutant (lane 1). 50-µg total cell lysates were used to perform Western blot analysis with antibodies specific for Skp2 (Skp2 H-435; Santa Cruz Biotechnology), p27 (p27 C-19; Santa Cruz Biotechnology), tuberin (tuberin C-20; Santa Cruz Biotechnology), and α-tubulin (Ab-1; Oncogene).

B, HEK 293 cells were transfected with an empty control vector (lane 1) or Skp2 (lane 2) or were co-transfected with Skp2 and TSC2 (lane 3) (left side). For the right side (lanes 4–6), the same experiments were performed using a double DNA amount of Skp2 plasmid per cell. 50-µg total protein lysates were analyzed for protein expression with antibodies specific for tuberin (tuberin C-20; Santa Cruz Biotechnology), Skp2 (Skp2 H-435; Santa Cruz Biotechnology), p27 (p27 C-19; Santa Cruz Biotechnology), and α-tubulin (Ab-1; Oncogene) (upper panel). In addition, protein extracts of these experiments were used to perform immunoprecipitations (IP) with an anti-p27 antibody (p27 C-19; Santa Cruz Biotechnology) and immunoblotting using anti-Skp2 (Skp2 H-435; Santa Cruz Biotechnology), anti-tuberin (tuberin C-20; Santa Cruz Biotechnology), and an anti-p27 antibody (p27 C-19; Santa Cruz Biotechnology). IgG L bands are shown to demonstrate equal immunoprecipitation levels (bottom). wt, wild type.

C, HEK 293 cells were transfected with Skp2 (lane 1) or co-transfected with Skp2 and TSC2 (lane 2). 500-µg protein extracts were used to perform immunoprecipitations (IP) with an anti-p27 antibody (p27 C-19; Santa Cruz Biotechnology) and immunoblotting using anti-cdk2 (M-2; Santa Cruz Biotechnology). In addition, 50-µg total cell lysates of these experiments were used for immunoblot detection with an anti-cdk2 antibody (M-2; Santa Cruz Biotechnology) to analyze cdk2 expression (bottom). wt, wild type.
antibody followed by immunodetection of Skp2 demonstrated that expressing a higher level of Skp2 increases its amount in the p27 immunoprecipitate. In the control, only one Skp2-specific band, which represents the endogenous form, is detected in the p27-specific immunoprecipitate (Fig. 5B, lane 1, bottom). Upon Skp2 overexpression in the p27-specific immunoprecipitations, an additional second band occurs above the endogenous Skp2 because the ectopic FLAG-tagged Skp2 is slightly larger than the endogenous form (Fig. 5B, lane 2, bottom). Co-overexpression of tuberin triggers a down-regulation of ectopic Skp2 bound to p27 (Fig. 5B, lane 3, bottom). In addition, we performed the same experiments using double the amount of Skp2 plasmid DNA per cell. Western blot analyses demonstrated that whereas the level of overexpressed tuberin was comparable with that in the first experiment, the overexpression levels of Skp2 were 2-fold higher (Fig. 5B, compare left and right sides). The intensity of the slower running Skp2-specific band in the p27 immunoprecipitations was found to be relative to the expression level of ectopic Skp2 (Fig. 5B, compare left and right sides). This finding further confirmed the specificity of this band. This comparison also allows the conclusion that the same amount of tuberin can still affect the interaction of p27 with Skp2 in cells with increased levels of Skp2 expression.

These data allow the conclusion that tuberin is able to sequester Skp2 from p27. Although interesting, this effect is not necessary for our model of tuberin’s function. The observation that tuberin up-regulates p27 in the same cell in which the amount of p27 bound to Skp2 remains equal (as in the endogenous system; Fig. 4B) already proves that tuberin protects a high amount of the p27 protein from binding to Skp2 and from degradation.

Still, this finding would not necessarily explain the effects of tuberin on cell cycle progression. Because earlier studies demonstrated that tuberin is involved in the regulation of cdk2 activity (11), it was of interest to investigate whether the stabilized p27 is able to bind cdk2. The comparison of HEK 293 cells overexpressing Skp2 alone with HEK 293 cells co-overexpressing Skp2 together with TSC2 revealed that ectopic tuberin is able to trigger an up-regulation of p27/cdk2 complex formation even in cells co-overexpressing Skp2 (Fig. 5C). An up-regulation of the interaction of p27 with cdk2 upon high levels of tuberin in cells not overexpressing Skp2 has already been shown in Fig. 4A. Taken together, these data provide evidence that tuberin does the following: 1) actively sequesters p27 from Skp2; 2) protects p27 from the Skp2-mediated degradation via the proteasome; 3) triggers an up-regulation of p27 bound to cdk2; 4) down-regulates cdk2 activity; and 5) thereby negatively regulates cell cycle progression.

**Tuberin Negatively Regulates Skp2-mediated Cell Cycle Progression**—A major expectation of the model presented here is that the overexpression of Skp2 leads to the enhanced degradation of p27 (as shown in Fig. 5, A and B) and promotes cell cycle progression, whereas the co-overexpression of tuberin under these circumstances should delay or even off-set these effects of Skp2. Whereas with respect to the regulation of p27 degradation we have already shown that tuberin can abolish the effects of Skp2 (Fig. 5, A and B), tuberin’s capacity to affect Skp2-mediated cell cycle regulation was not yet proven. It is known that Skp2 alone can promote cell cycle progression, allowing Skp2 to join the restricted club of proteins consisting of Myc, E2F, and cyclinE/cdk2, which are endowed with this property. In addition to p27, Skp2 allows other substrates, such as E2F-1, to be marked for degradation. Accordingly, Skp2 appears to be well suited to be one of the key rate-limiting activities for the regulation of cell cycle progression (reviewed in Ref. 26). We overexpressed Skp2 alone or co-overexpressed Skp2 together with TSC2 in MEFs (Fig. 6A) in which the interaction of tuberin and p27 has been demonstrated (Fig. 1D). G418-selected pools of the transfected cells were harvested, reseeded with equal cell numbers, and kept growing for 7 days. Compared with the empty vector control, ectopic Skp2 had an impressive effect on the cell cycle progression, which

![Fig. 6. Tuberin attenuates Skp2-mediated cell cycle progression. A, MEFs were either transfected with the empty control vector (lane 1) or Skp2 (lane 2) or were co-transfected with Skp2 and TSC2 (lane 3). After 48 h, overexpression was confirmed by Western blot analysis using an anti-tuberin antibody (tuberin C-20; Santa Cruz Biotechnology) and an anti-Skp2 antibody (Skp2 H-435; Santa Cruz Biotechnology). In this non-transformed cell line, endogenous Skp2 is under the detection level. *wt*, wild-type. B, MEFs were either transfected with the empty control vector or Skp2 or were co-transfected with Skp2 and TSC2 (see also above). G418 selection for transfected cells was started 24 h after transfection. Starting with equal cell numbers (harvesting and reseeding), selected cell pools were grown under further selection for 7 days, and cell numbers were determined on the CASY cell counter and analyzer. The data are given in percentages relative to the mean value of the controls, set as 100%. The cell numbers are presented as the mean ± S.D. For the difference between the control cells and the Skp2-overexpressing cells, the p value was 0.0016. For the difference between the Skp2-overexpressing cells and the cells co-overexpressing Skp2 together with TSC2, the p value was 0.0021. *wt*, wild-type.](http://www.jbc.org/content/344/31/48713/F6.large.jpg)
FIG. 7. Tuberin stabilizes p27 via regulating the interaction of Skp2 with p27. Skp2 binds p27 and induces its degradation via the proteasome. Accordingly, up-regulation of Skp2 positively regulates cell cycle progression. Binding of tuberin to p27 sequesters Skp2 from p27, triggering p27 stabilization. High levels of tuberin cause cell cycle arrest.

was completely reversed by tuberin. In the past, we and others have used this approach to demonstrate the negative effects of tuberin on cell cycle progression (23, 24). These effects could also be seen in cells co-overexpressing Skp2 and tuberin (Fig. 6B). These data demonstrate tuberin to be a potent counter-agent against Skp2 in regulating the mammalian cell cycle.

**DISCUSSION**

Using our data, we have now developed a model of the counteracting potencies of the F-box protein Skp2 and the tumor suppressor protein tuberin (Fig. 7). Up-regulation of Skp2, if done upon ectopic overexpression or during G1 in the normal cell cycle, triggers p27 degradation and cell cycle progression. High levels of ectopic tuberin arrest the cell cycle by protecting p27 from Skp2-mediated degradation.

**The Molecular Events Associated with the Disease TSC—**

Loss of functional tuberin or hamartin triggers the molecular development of TSC. This loss leads to an intracellular increase in GTP-bound Rheb, which, in turn, leads to activation of the mammalian target of the rapamycin/S6 kinase/4E-binding protein signaling pathway (reviewed in Refs. 6 and 7). In addition, cells lacking functional tuberin or hamartin, such as the cells that give rise to the hamartomas of TSC patients, may fail to arrest because the cdk inhibitor p27 is inactivated. Tuberin has been shown to regulate p27 via affecting its protein stability and regulating its localization (11). p27 cannot mediate the same functions in tuberin-negative cells as in tuberin-positive counterparts, and this could be due to either deregulated p27 stability or localization or both. It is known that these two processes are differently regulated. Whereas p27 degradation depends on its phosphorylation by cdk2, the localization of p27 (cytoplasm versus nucleus) is regulated by its phosphorylation via other kinases such as, e.g., Akt, and by association of p27 with Jab-1 (Jun activation domain-binding protein-1) and/or with 14-3-3 (22, 25). To date, it remains unclear whether the molecular mechanisms of p27 localization and degradation are connected. It has been shown that the loss of tuberin or hamartin triggers p27 protein down-regulation in vivo. Astrocytes and fibroblasts of TSC1+/− or TSC2+/− mice as well as astrocytes of astrocyte-specific TSC1 conditional knock-out mice exhibit decreased p27 expression (27, 28). Investigations of the localization of p27 in TSC-associated lesions are still missing. Here, we demonstrate that tuberin binds to p27 and hinders the SCF component Skp2 in binding p27 and in inducing its degradation by the proteasome. This molecular elucidation of tuberin’s effects on p27 protein stability strengthens the argument that p27 could also be considered a target for hamartoma therapeutics in TSC (29). The molecular mechanism of tuberin’s effects on p27 localization still remains elusive and is currently under investigation in our laboratory. Recently, we found that specific natural occurring disease-causing mutants can still regulate p27 protein stability and proliferation (30). In addition to cell cycle control, p27 has been implicated in the regulation of a wide variety of different cellular processes such as apoptosis, cell growth, or tumorigenesis (25, 29, 31). These findings make it tempting to speculate that specific disease-causing mutations in the TSC genes could cause a loss of the proper control of p27 stability and proliferation, whereas other TSC gene mutations could trigger a loss of the proper control of p27 localization, which could be involved in the regulation of apoptosis. The fact that the same TSC gene mutant can behave differently with respect to different cellular processes has recently been demonstrated by our finding that naturally occurring disease-causing TSC2 mutants, which can still regulate p27 stability and proliferation, lost the capacity to regulate cell size in mammalian cells (23).

*p27 as a Tumor Suppressor—*Mouse models have provided evidence that the loss of p27 is involved in the development of cancers. Furthermore, the p27 protein has emerged as a prognostic indicator in many different types of human cancer, including those of the colon, breast, prostate, lung, and ovary as well as in brain tumors and lymphomas, with abnormally low protein levels indicating more aggressive tumors with poorer prognosis. p27 may contribute to tumor development by either increasing the proliferation of cells or decreasing their apoptosis. The deregulation of p27 in tumors is due to enhanced proteolysis (neither loss or mutation of the p27 gene nor mRNA
deregulation can be detected). This observation suggests that proteins involved in p27 degradation may have oncogenic properties. Indeed, Skp2 levels have been shown to correlate with the grade of malignancy in several human tumors (reviewed in Refs. 25 and 31). From our data one might speculate that loss or mutation of the TSC genes could contribute to the regulation of p27 in different human cancers. Indeed, TSC gene mutations have recently been demonstrated in sporadic bladder cancer. Additionally, deletions in the TSC gene regions are found with significant frequency in ovarian and gall bladder carcinoma. Moreover, deletions in the TSC1 gene regions are found in about 10% of sporadic bladder tumors (25). From our data one might speculate that loss of p27 in different human cancers. Indeed, TSC gene mutations are now merited (32).

Regulation of the Activity of an SCF Ligase against a Substrate—Cell cycle control is crucially dependent on the ubiquitin-mediated degradation of key regulatory proteins. From the onset of anaphase until the subsequent G1/S transition, the ubiquitin-mediated degradation of key regulatory proteins. From the anaphase-promoting complex/cyclosome (APC/C) mediates degradation of a wide variety of substrates such as cyclin A and B. Another class of protein-ubiquitin ligases is known as the anaphase-promoting complex/cyclosome (APC/C) mediates degradation of a wide variety of substrates such as cyclin A and B. Another class of protein-ubiquitin ligases is known as the Skp1/cullin/F-box protein-SCF ligase against a substrate, i.e., binding of the substrate to a specific protein inhibits its interaction with the E3 ubiquitin ligase. It will be of great interest in the future to investigate whether other proteins harbor such a potency and whether other substrates are regulated this way.

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