[CANCER RESEARCH 62, 5645–5650, October 15, 2002]

Advances in Brief

Activated Mammalian Target of Rapamycin Pathway in the Pathogenesis of Tuberous Sclerosis Complex Renal Tumors[1]

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Abstract

Disruption of the *TSC1* or *TSC2* gene leads to the development of tumors in multiple organs, most commonly affecting the kidney, brain, lung, and heart. Recent genetic and biochemical studies have identified a role for the tuberous sclerosis gene products in phosphoinositide 3-kinase signaling. On growth factor stimulation, tuberin, the TSC2 protein, is phosphorylated by Akt, thereby releasing its inhibitory effects on p70S6K. Here we demonstrate that primary tumors from tuberous sclerosis complex (TSC) patients and the Eker rat model of TSC expressed elevated levels of phosphorylated mammalian target of rapamycin (mTOR) and its effectors: p70S6K, S6 ribosomal protein, 4E-BP1, and eIF4G. In the Eker rat, short-term inhibition of mTOR by rapamycin was associated with a significant tumor response, including induction of apoptosis and reduction in cell proliferation. Surprisingly, these changes were not accompanied by significant alteration in cyclin D1 and p27 levels. Our data provide *in vivo* evidence that the mTOR pathway is aberrantly activated in TSC renal pathology and that treatment with rapamycin appears effective in the preclinical setting.

Introduction

TSC[3] is an autosomal dominant syndrome associated with the multiorgan development of benign and occasional malignant tumors most commonly affecting the central nervous system, kidney, and skin (1). Lesions such as cortical tubers, subependymal giant cell astrocytoma, cardiac rhabdomyomas, and renal AML often exhibit abnormal patterns of differentiation along with deregulated cell growth and proliferation. The biochemical bases of these pathological alterations are not well understood, but genetic studies in *Drosophila* indicate that the two genes implicated in tuberous sclerosis, *TSC1* and *TSC2*, participate in the control of cell size via the insulin/p70S6K pathway (reviewed in Ref. 2). Epistasis experiments demonstrate that dTsc1 and dTsc2 act upstream of dS6K and downstream of dAkt. Recent biochemical analyses confirmed that tuberin, the *TSC2* gene product, is a substrate of Akt and can modulate PI_3K-dependent activation of p70S6K (3, 4). Phosphorylation of tuberin by Akt reduces the stability of tuberin and thereby releases its inhibitory function on p70S6K signaling. It has also been shown that disease-causing *TSC2* mutations can produce a reduced state of tuberin phosphorylation that causes it to interact less stably with the *TSC1* product, hamartin (5). Beyond this, *TSC1* and *TSC2* have been implicated in other molecular pathways, including regulation of low-molecular weight GTPases (Rap1, Rab5, Rho), p27 stability, and steroid-dependent transcription (reviewed in Ref. 6). As a large molecular complex, TSC1-TSC2 could potentially mediate multiple pathways related to cell growth, proliferation, differentiation, and migration, all of which are relevant to TSC biology.

In this study, we examined the relevance of the PI3K/TSC2/S6K pathway in the pathogenesis of TSC renal manifestations. p70S6K is a key effector of the PI_3K pathway whose activity is regulated by sequential phosphorylation by multiple upstream kinases (7). Phosphorylation of the critical residue, Thr^{229}, in the activation loop of p70S6K is mediated by PDK1 and is most efficient after prephosphorylation at Thr^{389} by mTOR and at the COOH-terminal autoinhibitory domain by various kinases (7). On activation, p70S6K phosphorylates S6 ribosomal protein to regulate translation of 5′-TOP mRNA and ribosome biogenesis. Binding of mRNA with the 40S ribosomal subunit is also under the control of the eIF4F complex, consisting of eIF4E, eIF4A, and eIF4G. Stimulation of protein synthesis by amino acids releases eIF4E from its inhibitory partner, 4E-BP1, on phosphorylation by mTOR (8). The latter cooperates with the PI_3K pathway to coordinate cellular responses to growth factors, nutrients, and energy sources. Conserved through evolution, TOR has been shown to control cell size by regulating protein synthesis mediated through downstream targets, p70S6K and 4E-BP1.

The importance of the mTOR pathway in human pathology is reflected in the overexpression of p70S6K in a subset of breast cancers and its correlation with a poor prognosis (9). Moreover, recent clinical studies have reported antitumor response to rapamycin and its ester derivatives. Rapamycin is a microbial product that binds the intracellular receptor FKBP12 to specifically inhibit mTOR activity (10). We propose that the loss of tuberin function leads to activation of the mTOR pathway in TSC-related renal tumors and that inhibition of mTOR signaling brings about reversal of the tumor phenotype. In primary renal tumors derived from TSC patients and the Eker rat model of TSC, multiple mTOR effectors and mTOR itself were found to be highly phosphorylated. Treatment with rapamycin in the Eker rat elicited a significant biochemical and histological tumor response in keeping with the hypothesis that mTOR is a relevant target for therapeutic intervention in TSC patients.

Materials and Methods

Antibodies and Chemicals. The antibody for PHAS-I (4E-BP1) was purchased from Zymed (San Francisco, CA), Kip1 (p27) was from Transduction Laboratories (Los Angeles, CA), cyclin D1 was from Rockland (Gilbertsville, PA), tuberin C20 was from Santa Cruz Biotechnology (Santa Cruz, CA), actin was from Sigma (St. Louis, MO), and PCNA was from DAKO (Carpinteria, CA). Antigelsolin antibody was a gift of David Kwiatkowski (Brigham and Women's Hospital, Boston, MA). All other antibodies were purchased from Cell Signaling (Beverly, MA). Rapamycin was purchased from Calbiochem (La Jolla, CA). An In-Situ Cell Death Detection Kit (peroxidase) with a 3,3′-diaminobenzidine substrate was obtained from Roche Diagnostics (Indianapolis, IN). Secondary antibodies and electrochemiluminescence reagents were purchased from Amersham Pharmacia Biotech (Piscataway, NJ). The

Received 8/1/02; accepted 9/3/02.

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[1] This work was supported by NIH Grant CA77882 and the Tuberous Sclerosis Alliance.

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[3] The abbreviations used are: TSC, tuberous sclerosis complex; AML, angiomyolipoma; PI_3K, phosphoinositide 3-kinase; mTOR, mammalian target of rapamycin; IHC, immunohistochemistry; PCNA, proliferating cell nuclear antigen; RCC, renal cell carcinoma; LOH, loss of heterozygosity; TUNEL, terminal deoxynucleotidyl transferase (TdT)-mediated nick end labeling.

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Elite ABC kits, 3,3′-diaminobenzidine, and Hematoxylin QS were purchased from Vector Laboratories (Burlingame, CA). Eosin was obtained from Richard Allen Scientific (Kalamazoo, MI).

Animals. The Eker rat strain harboring a germ-line *TSC2* mutation was as described previously (11). Fischer male carriers were identified by genotyping and housed and fed *ad libitum* until the age of 12 months, at which point rapamycin was injected i.p. once daily for 3 consecutive days. The control animal was given the same volume and concentration of DMSO-vehicle, and the treated rats were given rapamycin at three dose levels (0.16, 0.4, and 1 mg/kg). Animals were sacrificed 24 h after the last injection, and tissues were procured for IHC and Western blot analysis. All work related to animals was in accordance with the protocol approved by the Animal Care Committee, University of Washington, Seattle.

IHC. Kidney samples were fixed in formalin and paraffin embedded. Five-μm sections were deparaffinized, rehydrated, and washed with PBS. After antigen retrieval in 10 mM sodium citrate (pH 6.0) and quenching of endogenous peroxidase activity with 1% H_2O_2, samples were blocked with 5% normal goat serum before incubation with primary antibodies overnight at 4°C. Negative controls were treated with 5% normal goat serum without the primary antibodies. Signals were processed according to the supplied protocol (Elite ABC Kit). Slides were counterstained with Hematoxylin QS, dehydrated, and mounted using Permount (Fischer Scientific, Santa Clara, CA). For the cell proliferation index, $PCNA^+$ tumor cells were counted from 10 random, non-overlapping high-power fields within the tumors, and the results were expressed as a percentage of the total number of tumor cells counted in the same fields.

Western Blotting. Tissues were homogenized in ice-cold radioimmunoprecipitation assay buffer [1% NP40, 1% sodium deoxycholate, 0.1% SDS, 0.15 M NaCl, 10 mM Tris (pH 7.2), 0.025 M β-glycophosphate (pH 7.2), 2 mM EDTA, and 50 mM sodium fluoride] with protease and kinase inhibitors [0.05 mM 4-(2-aminoethyl)benzenesulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml pepstatin, 1 mM orthovanadate, 10 μg/ml leupeptin, 1 mM microcystin LR]. The protein concentration was measured using the BCA Protein Assay (Pierce, Rockford, IL). Equal amounts of protein were separated by SDS-PAGE, transferred to Immobilon-P membranes (Millipore, Bedford, MA), and blotted with antibodies according to the manufacturer's recommendations, as described previously (5).

Results

Expression of Phospho-p70S6K in Human TSC Renal Pathology. Patients with TSC are predisposed to the development of two forms of renal tumors: RCC and AML (1). To determine the status of the p70S6K pathway in these tumors, IHC was performed using antibodies that specifically recognize phosphorylated p70S6K at Thr^{389}, a modification that is necessary for activation of the protein, and phosphorylated mTOR at Ser^{2448}, a site that has been associated with activity (7, 12). Three cases of RCC derived from TSC patients were examined, all demonstrating elevated levels of phospho-mTOR and phospho-p70S6K expression in the tumor cells compared with adjacent uninvolved kidney tissue (Fig. 1*A*). The staining pattern of p70S6K was cytoplasmic, whereas the mTOR signal could be seen in the cytoplasm and nucleus, consistent with previous localization studies (13, 14). We did not find elevated levels of phospho-p70S6K in a few sporadic RCCs examined (data not shown); sporadic clear-cell RCCs are known to involve pathways other than TSC1 and TSC2 (15).

The second and more common form of renal pathology in TSC patients is AML. These tumors contain variable proportions of three histological components: adipocytes, smooth muscle cells, and vascular structures. In five of six AMLs from TSC patients, we found robust expression of phospho-p70S6K compared with adjacent tissues (Fig. 1*B*). Specifically, staining was uniformly identified in the smooth muscle and lipomatous components, whereas the vascular structures showed a heterogeneous pattern. Many endothelial lined structures were surrounded by phospho-p70S6K-positive smooth muscle-like cells that were contiguous with the myolipomatous components of the tumor (Fig. 1*B*, *left*). However, vessels with discrete walls were found to stain minimally for phospho-p70S6K (Fig. 1*B*, *right*). The latter structures may represent preexisting or reactive vessels. A sporadic AML was also found to aberrantly express phospho-p70S6K (data not shown). This is consistent with the identification of LOH at the *TSC2* locus in sporadic AMLs and suggests a common pathogenic mechanism of deregulated p70S6K activity in both the sporadic and familial forms of AML (16).

Within the normal kidney, discrete expression of phospho-p70S6K was detected in the distal tubules and some collecting ducts; the proximal tubules and glomeruli lacked immunoreactivity (Fig. 1*C*). Approximately 5% of the cells in the normal kidney expressed detectable levels of phospho-p70S6K. Because the latter has been implicated in cell size control by regulating 5′-TOP translation, it was unexpected to find that phospho-p70S6K-positive distal tubular cells were consistently smaller than those in adjacent proximal tubules.

Collectively, IHC analyses of human TSC renal pathology showed overexpression of activated mTOR and p70S6K. To further investigate the functional role of this pathway in tumorigenesis, we examined spontaneous renal tumors derived from the Eker rat strain, a well-characterized animal model of TSC (11).

Expression of mTOR Effectors in Primary RCC of the Eker Rat. The Eker rat carries a germline mutation of the *TSC2* gene as a result of a retrotransposition of an endogenous IAP element. As such, the Eker *TSC2* allele behaves as a null mutation. Spontaneous renal cortical epithelial tumors in these animals have been shown to possess biallelic inactivation of *TSC2* through LOH or nonsense or missense mutations (reviewed in 17). In addition to p70S6K, mTOR also regulates 4E-BP1 in controlling cap-dependent translation (8). Using antibodies for 4E-BP1 and phospho-S6, a substrate for p70S6K, we analyzed the expression of these proteins in a panel of five primary tumor lysates by Western blotting. Compared with normal kidney, all tumors showed marked phosphorylation of ribosomal protein S6 and 4E-BP1 (Fig. 2*A*). These tumors also have elevated levels of cyclin D1 accompanied by minimal expression of p27 and phospho-Akt. Of the five tumors, three (tumors 2, 4, and 5) had lost expression of tuberin, and the remaining two were expected to possess aberrant forms of the protein as a result of missense mutations. Our results suggest that effectors downstream of mTOR were activated in the Eker renal lesions, consistent with the IHC findings in human TSC tumors.

To examine the distribution of these proteins in the Eker rat kidney, immunostaining was performed using phospho-specific antibodies in paraffin-embedded tissues. Intense, uniform staining for phospho-S6 was found in tumors of all sizes compared with adjacent renal parenchyma (Fig. 2*B*). Lesions in their earliest form with only a few cells were also decorated with phospho-S6 immunoreactivity (Fig. 2*F*). This suggests that the mTOR pathway is activated in the primary stages of renal tumorigenesis in the Eker rat. Other downstream targets of mTOR, including p70S6K, 4E-BP1, and eIF4G, were also expressed in their phosphorylated forms, although their patterns showed greater degree of intratumoral heterogeneity (Fig. 2, *C–E*). Conversely, pathways such as that of mitogen-activated protein kinase did not appear affected, as shown by the absence of phospho-Erk expression in the tumors based on IHC (Fig. 2*G*) and Western blotting (data not shown). The staining pattern of phospho-S6 in the nontumor portion of the Eker rat kidney was specific for cells within the distal tubules, similar to that found in wild-type kidneys in rats (data not shown).

Inhibition of the mTOR Pathway in RCC. Recent studies have shown that elevated levels of phospho-p70S6K and phospho-S6 in cells lacking TSC1 or TSC2 can be inhibited by rapamycin *in vitro* (18, 19). Similarly, we have observed that rat embryo fibroblasts in

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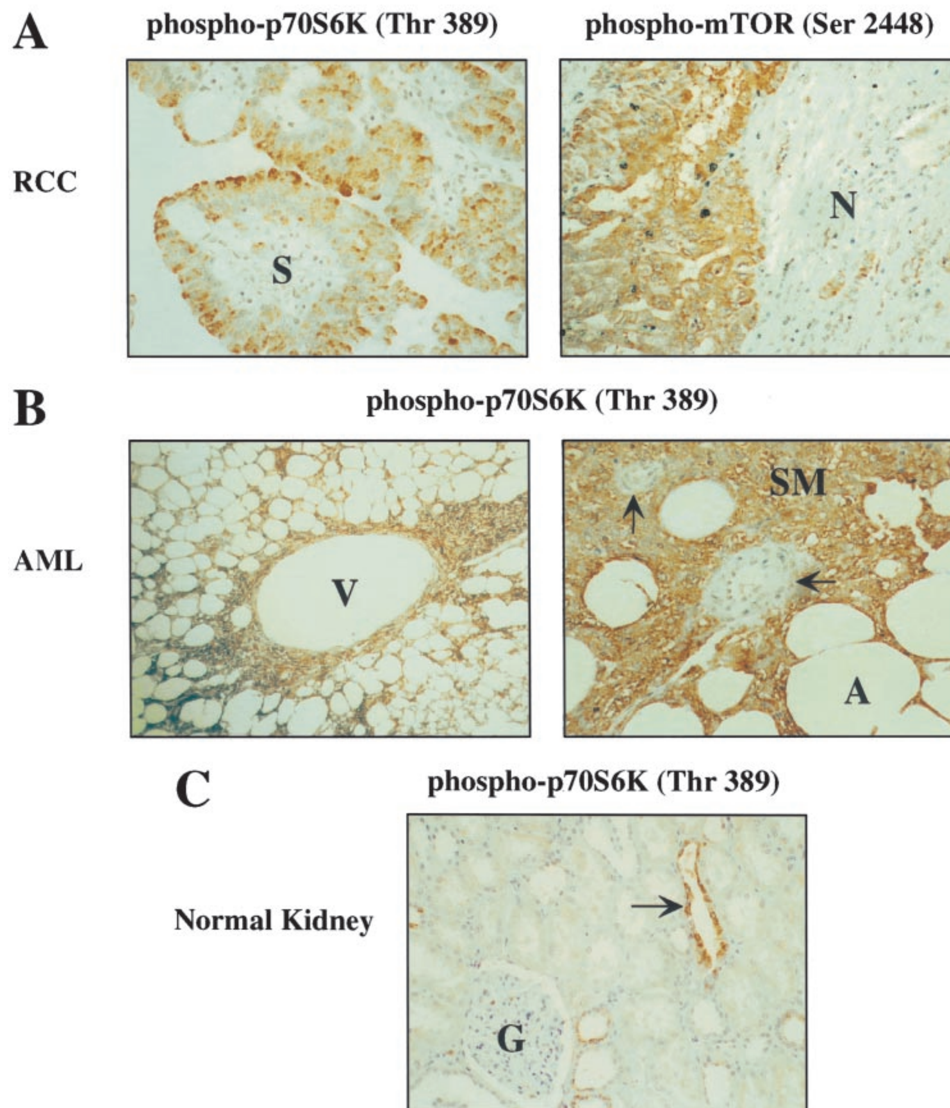

Fig. 1. Expression of mTOR and p70S6K in human TSC tumors and normal kidney. *A*, papillary RCC from a TSC patient stained with phospho-specific antibodies for p70S6K (Thr^{389}) and mTOR (Ser^{2448}). Note negatively stained stromal cells (*S*) and adjacent kidney tissue (*N*) compared with *brown-stained* tumor cells (magnification, ×400 for *left panel*, ×200 for *right panel*). *B*, TSC-related AMLs stained with phospho-p70S6K antibody. *Left panel* shows uniform staining in all three cellular components, including the central vessel (*V*; magnification, ×200). *Right panel* shows negative-staining vessels (*arrows*; magnification, ×400). *A*, adipocyte; *SM*, smooth muscle. *C*, normal human kidney stained with phospho-p70S6K antibody. *Arrow* indicates prominently stained distal tubule (magnification, ×200). *G*, glomerulus.

the absence of tuberin have constitutive activation of mTOR effectors, including 4E-BP1, eIF4G, S6K, and S6, as seen in the Eker rat renal tumors; all of these effectors can be down-regulated by rapamycin and LY294002 but not by Wortmannin or PD98059 (data not shown).

To investigate the dependence of *in vivo* tumor growth on the mTOR pathway, we treated animals with rapamycin and monitored tumor responses. After three daily i.p. doses of rapamycin at three dose levels, none of the animals appeared ill or behaved abnormally. On the fourth day, the Eker rats were sacrificed and the kidney tumors were analyzed for phosphorylation of the mTOR effectors by use of phospho-specific antibodies. At the highest rapamycin dose (1 mg/kg), we did not find tumors large enough for tissue homogenization. On Western blotting, the level of phospho-S6 in the tumors treated with vehicle only was highly elevated compared with the adjacent kidney tissue (Fig. 3*A*). Treatment with rapamycin dramatically reduced phospho-S6 expression in the tumors even at the lowest dose (0.16 mg/kg). Phosphorylation of 4E-BP1 was partially suppressed by rapamycin as shown by the increasing intensity of the faster mobility band with higher doses. Surprisingly, the levels of cyclin D1 and p27 expression, when corrected for protein loading, did not change significantly with rapamycin treatment (Fig. 3*A*). These findings correlated well with IHC. Fig. 3*B* shows specific reduction of phospho-S6 reactivity with rapamycin treatment without significant change in the overall expression of S6 in the renal tumors. To rule out nonspecific response to rapamycin, serial sections were stained with gelsolin, an actin-binding protein that is expressed in intercalated cells of the distal tubules and has been shown previously to be a marker for TSC-related pathology (20). The level of gelsolin immunoreactivity did not change significantly after rapamycin treatment (data not shown). In the normal kidney adjacent to the tumors, phospho-S6 immunostaining of the distal tubules was also decreased with rapamycin administration.

To determine tumor response to rapamycin, we examined the histology, cell proliferation, and apoptosis in the renal tumors. Vehicle control-treated lesions were indistinguishable from those that were untreated (data not shown). However, rapamycin-treated tumors showed significantly more condensed, fragmented, and pyknotic nuclei compared with vehicle-treated lesions (Fig. 3*B*, *H&E*). TUNEL staining confirmed the greater extent of apoptosis in the treated tumors compared with control (Fig. 3*B*). With increasing doses of rapamycin, the degree of apoptosis decreased as tumor necrosis increased. Lymphoid infiltration did not change significantly with rapamycin dose, but did correlate in a direct manner with tumor size. The percentage of $PCNA^+$ nuclei was used as an index of cell proliferation (Table 1). Rapamycin-treated tumors were associated with a smaller proportion of $PCNA^+$ cells. There was a highly

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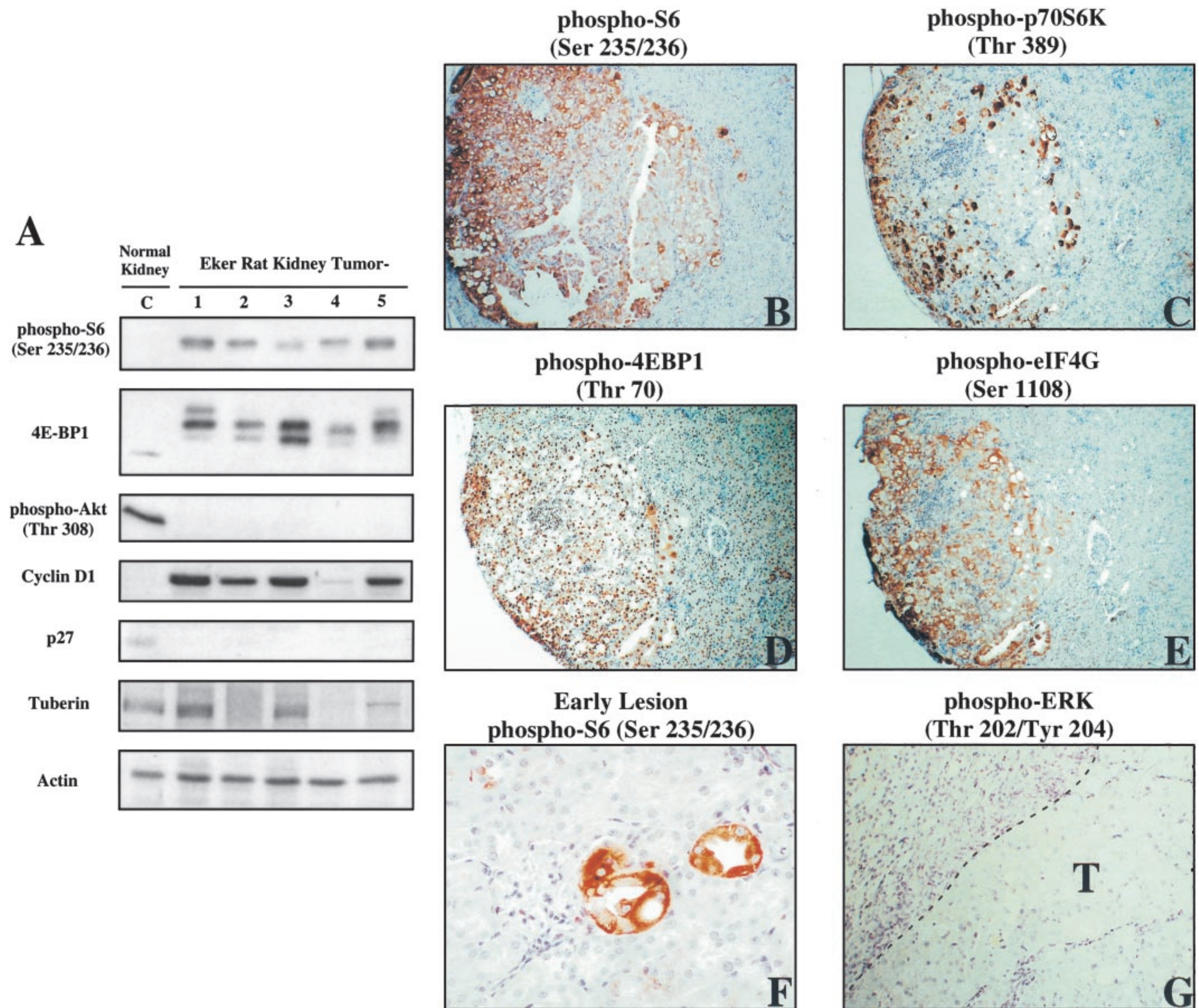

Fig. 2. mTOR effectors in the Eker renal tumors. *A*, Western blot analysis of primary tumor lysates showing expression of the indicated proteins. Actin was used as loading control. *B–G*, immunohistochemical staining of renal tumors with phospho-specific antibodies: *B*, S6 ($Ser^{235/236}$; magnification, ×100); *C*, p70S6K (Thr^{389}; magnification, ×100); *D*, 4E-BP1 (Thr^{70}; magnification, ×100); *E*, eIF4G (Ser^{1108}; magnification, ×100); *F*, S6 ($Ser^{235/236}$) staining of an early lesion (magnification, ×400); *G*, Erk (Thr^{202}/Tyr^{204}), *T*, tumor tissue (magnification, ×200).

significant inverse correlation between the rapamycin dose and the percentage of $PCNA^+$ cells.

Overall, rapamycin, even at the lowest dose administered, produced profound inhibition of p70S6K activity, which correlated with increased tumor cell death and necrosis. Importantly, rapamycin did not cause any significant histological alteration to the nontumor portions of the kidneys. If these observed effects are sustained, rapamycin is expected to be an effective therapy for the renal manifestations of TSC.

Discussion

In this study, we showed that tumors associated with *TSC* gene mutations are accompanied by activation of the mTOR pathway, including p70S6K, 4E-BP1, and eIF4G. In humans, RCC is an infrequent component of TSC, whereas AML is a common manifestation. Both types of tumors were shown to express phosphorylated mTOR/p70S6K and their substrates. These alterations appear specific to TSC because sporadic RCCs do not share the same immunohistological phenotype. Clear-cell and papillary RCCs are known to involve pathways independent of TSC1 or TSC2 (15). Interestingly, sporadic AMLs exhibit evidence of increased phospho-S6 expression consistent with earlier reports of *TSC1/2* LOH in these lesions (16). Immunostaining with the phospho-specific antibodies used in this study may aid in the classification of sporadic AMLs and RCCs with respect to their underlying pathogenesis. In the setting of TSC, abnormal "tumor" cells can be recognized by their expression of phosphorylated p70S6K, S6, or 4E-BP1. As potential surrogate markers of TSC pathology, further studies are needed to address their specificity.

The kidney tumors in the Eker rat have an immunophenotype similar to the human lesions, thus further validating the use of the Eker rat as a model of human TSC. Importantly, the fact that rapamycin treatment can down-regulate mTOR effectors and induce tumor response *in vivo* points to the biological dependence of renal lesions on the effects of the activated mTOR pathway. Of note, adjacent normal kidneys showed minimal cellular toxicity from rapamycin treatment. This is in agreement with the vast clinical experience in

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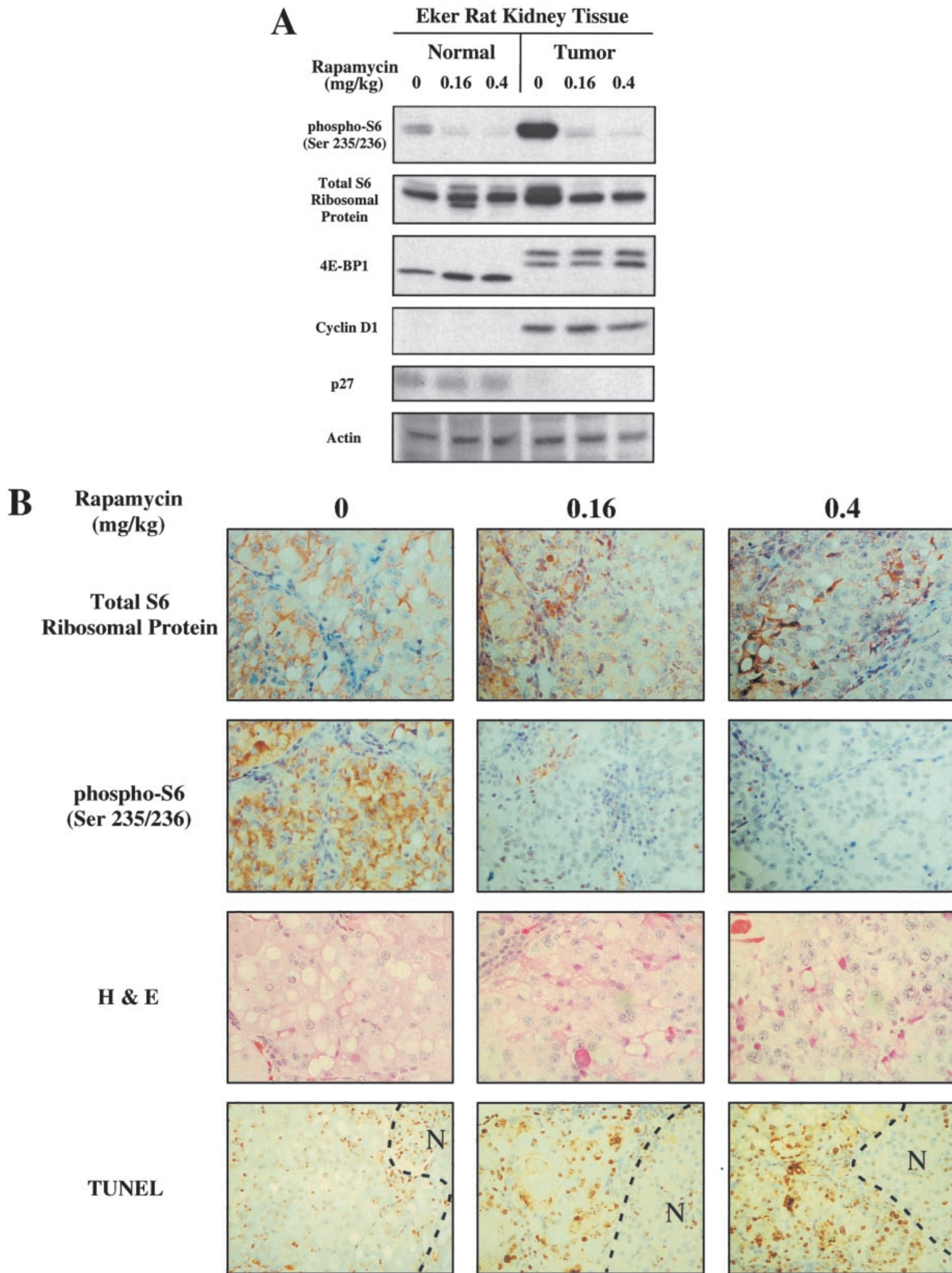

Fig. 3. Effects of rapamycin on Eker renal tumors. *A*, Western blot analysis of primary tumor lysates showing biochemical responses to i.p. rapamycin. The tumor samples were compared with adjacent normal kidney tissue for each dose level. Actin served as loading control. *B*, representative sections of renal tumors from animals treated with various doses of rapamycin stained for total S6, phospho-S6 ($Ser^{235/236}$), H&E, and apoptosis using the TUNEL kit. *N*, normal kidney.

using rapamycin to prevent rejection in renal transplant patients. The "selective" antitumoral effects on the Eker renal tumors seen after a short exposure to rapamycin suggest mechanisms in addition to regulation of protein synthesis. The rapid induction of apoptosis/necrosis in the tumors was unexpected on the basis of inhibition of translation alone. A recent study showed that p70S6K signals cell survive by inactivating BAD through phosphorylation of Ser^{136} (21). Hence, acute down-regulation of p70S6K in tumor cells is expected to promote apoptosis. Furthermore, rapamycin has been shown to be a potent cell cycle inhibitor by down-regulating cyclin D (22). Given

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Table 1 *PCNA staining in Eker renal tumors*

Rapamycin	No. of cells counted Positive	Negative	% positive[a]
Control	268	238	53
0.16 mg/kg	97	307	24
0.4 mg/kg	88	581	13
1.0 mg/kg	59	683	8

[a] $\chi^2 = 353$; $P < 0.0001$. There is a highly significant trend between the proportion of $PCNA^+$ cells and rapamycin dose.

that the half-life of the cyclin D family of proteins is short (~2 h), the relatively stable levels of cyclin D and p27 in the treated renal tumors do not support this mechanism as the cause of reduced cell proliferation in this *in vivo* model. Alternatively, rapamycin may induce tumor response through an antiangiogenesis pathway. Both hypoxia-induced vascular proliferation and insulin-dependent stimulation of HIF-1 have been shown to be dependent on mTOR (23, 24). In addition, in murine models, rapamycin inhibited vascular endothelial growth factor response, angiogenesis, and tumor growth (25). Collectively, our findings implicate the mTOR pathway as a biologically relevant target in TSC-related tumors. Short-term pharmacological manipulation of mTOR activity can bring about significant antitumoral effects by promoting cell death and reducing cell proliferation by a cyclin D-independent pathway.

As a sensor of nutrients, growth factors, and ATP, mTOR serves a critical role in regulating the translational machinery and, in doing so, affects cellular responses to growth, proliferation, and differentiation, all of which are abnormally manifested in TSC lesions. To date, little is known about the upstream regulators of mTOR. Protein kinase B, also known as Akt, has been shown to phosphorylate mTOR Ser^{2448} *in vitro*, but its biological relevance remains unclear because disruption of this site does not affect mTOR signaling and deletion of this region enhances its kinase activity (26). However, insulin-induced phosphorylation of 4E-BP1 was shown to be Akt-mediated and dependent on mTOR activity (27). Furthermore, a functional link between Ser^{2448} phosphorylation and muscle hypertrophy/atrophy was noted *in vivo* (12). In this study, sustained phosphorylation of mTOR (Ser^{2448}) and its downstream targets in TSC pathology support a role of TSC2 in regulating mTOR downstream of Akt. Consistent with this model, expression of tuberin was shown in a recent study to suppress mTOR activation of p70S6K and to modulate the level of Ser^{2448} mTOR expression (28). However, current data do not distinguish between a model where tuberin acts upstream of mTOR *versus* one that converges on a common downstream target.

In summary, activated mTOR signaling in TSC renal pathology provides evidence that this pathway, among others, is relevant to their pathogenesis. At least some of the classic TSC cellular phenotype (*e.g.,* abnormal cell size) can now be explained by this mechanism. Encouragingly, induction of an *in vivo* response to short-term rapamycin treatment in spontaneous renal tumors of the rat model lend support to its use in the clinical setting.

Acknowledgments

We thank Jean Campbell and members of Dr. Yeung's laboratory for critical reading and suggestions. We are grateful to David Ewalt, Lisa Henske, and Laura Finn for providing TSC-related pathology. This work is dedicated to Dr. Alfred Knudson, Jr. on his 80th birthday.

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