Signaling Events Downstream of Mammalian Target of Rapamycin Complex 2 Are Attenuated in Cells and Tumors Deficient for the Tuberous Sclerosis Complex Tumor Suppressors

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Abstract

Mutations in the *TSC1* and *TSC2* tumor suppressor genes give rise to the neoplastic disorders tuberous sclerosis complex (TSC) and lymphangioleiomyomatosis. Their gene products form a complex that is a critical negative regulator of mammalian target of rapamycin (mTOR) complex 1 (mTORC1) and cell growth. We recently found that the TSC1-TSC2 complex promotes the activity of mTOR complex 2 (mTORC2), an upstream activator of Akt, and this occurs independent of its inhibitory effects on mTORC1. Loss of mTORC2 activity in cells lacking the TSC1-TSC2 complex, coupled with mTORC1-mediated feedback mechanisms, leads to strong attenuation of the growth factor–stimulated phosphorylation of Akt on S473. In this study, we show that both phosphatidylinositol 3-kinase–dependent and phosphatidylinositol 3-kinase–independent mTORC2 substrates are affected by loss of the TSC1-TSC2 complex in cell culture models and kidney tumors from both *Tsc2*$^{+/-}$ mice (adenoma) and TSC patients (angiomyolipoma). These mTORC2 targets are all members of the AGC kinase family and include Akt, protein kinase Cα, and serum and glucocorticoid-induced protein kinase 1. We also show that the TSC1-TSC2 complex can directly stimulate the *in vitro* kinase activity of mTORC2. The interaction between these two complexes is mediated primarily through regions on TSC2 and a core component of mTORC2 called Rictor. Hence, loss of the TSC tumor suppressors results in elevated mTORC1 signaling and attenuated mTORC2 signaling. These findings suggest that the TSC1-TSC2 complex plays opposing roles in tumor progression, both blocking and promoting specific oncogenic pathways through its effects on mTORC1 inhibition and mTORC2 activation, respectively. [Cancer Res 2009;69(15):6107–14]

Introduction

TSC1 and TSC2 (also called hamartin and tuberin, respectively) are encoded by the tumor suppressor genes mutated in tuberous sclerosis complex (TSC), a tumor syndrome characterized by neoplastic lesions most commonly affecting the brain, kidneys, skin, heart, and lungs (1). These proteins form a complex in which TSC1 stabilizes TSC2, and TSC2 acts as a GTPase-activating protein (GAP) for the Ras-related small G protein Rheb (2). Through its GAP activity, the TSC1-TSC2 complex inhibits the ability of Rheb to activate mammalian target of rapamycin (mTOR) complex 1 (mTORC1 or the mTOR-Raptor-mLST8 complex), a critical promoter of cell growth and proliferation (3). Signaling pathways, composed of several oncogenes and tumor suppressors, converge on the TSC1-TSC2 complex to properly regulate Rheb and mTORC1 (2). Among other downstream targets, mTORC1 phosphorylates the ribosomal S6 protein kinases (S6K1 and S6K2) on a site just COOH-terminal to their kinase domains, within a hydrophobic motif (F-X-X-F/Y-S/T-F/Y, where X is any amino acid) that is highly conserved among members of the AGC (protein kinases A, G, and C) family of protein kinases.

The mTOR kinase also exists in another multiprotein complex, mTOR complex 2 (mTORC2 or the mTOR-Rictor-mSin1-mLST8 complex; ref. 3). This complex is functionally distinct from mTORC1 and phosphorylates the hydrophobic motif on other members of the AGC kinase family, including Akt (S473), protein kinase Cα (PKCα; S657), and serum and glucocorticoid-induced protein kinase 1 (SGK1; S422; refs. 4–6). The phosphorylation of another conserved motif on Akt and PKCα, called the turn motif (T450 on Akt), is also dependent on mTORC2 (7, 8). Unlike mTORC1, the mechanisms of regulation of mTORC2 activity are poorly understood. Although the kinase activity of mTORC2 can be stimulated by growth factors, perhaps downstream of phosphatidylinositol 3-kinase (PI3K; refs. 9, 10), some functions of mTORC2, such as phosphorylation of PKCα or the turn motif on Akt, are not dependent on growth factor signaling (7, 8).

We recently made the surprising finding that the TSC1-TSC2 complex, while inhibiting mTORC1 signaling, promotes mTORC2 activity (10). We showed that the kinase activity of mTORC2 is attenuated in a variety of cell lines lacking the TSC1-TSC2 complex, and reciprocally, this activity can be stimulated by TSC2 overexpression. Importantly, these effects of the TSC1-TSC2 complex on mTORC2 activity are independent of its regulation of Rheb and mTORC1. Most surprising was the finding that the TSC1-TSC2 complex can physically associate with components of mTORC2 but not those unique to mTORC1. We report here that several phosphorylation events mediated by mTORC2 are disrupted in TSC2-deficient cells and tumors. These include phosphorylation sites stimulated by growth factors and PI3K signaling as well as those that are constitutive and occur independent of PI3K. We also find that purified TSC1-TSC2 complex can stimulate the *in vitro* kinase activity of mTORC2 and that the interaction between the two complexes is mediated primarily through TSC2 and Rictor. Therefore, loss of the TSC tumor suppressors creates a rather unique molecular setting where Rheb-mTORC1 signaling is elevated and mTORC2 signaling is attenuated, and this is likely to account for the unique clinical features of the TSC disease relative to other tumor syndromes in which this pathway is corrupted.

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Materials and Methods

Cell culture, constructs, and immunoblots. HEK293 and mouse embryonic fibroblast (MEF) lines were maintained in DMEM with 4.5 g/L glucose containing 10% fetal bovine serum. The littermate-derived pair of *Tsc2*$^{+/+}$ and *Tsc2*$^{-/-}$ MEFs (both *p53*$^{-/-}$) was provided by Dr. D.J. Kwiatkowski and were described previously (11). The littermate-derived pair of *Rictor*$^{+/+}$ and *Rictor*$^{-/-}$ MEFs (also *p53*$^{-/-}$) was provided by Drs. D.A. Guertin and D.M. Sabatini (Massachusetts Institute of Technology). The isogenic pair of control and reconstituted *Tsc2*$^{-/-}$ *p53*$^{-/-}$ MEFs was generated as described previously (10).

All transfections were done using Polyfect (Qiagen) according to the manufacturer's protocol. The pcDNA3-FLAG-Rap1Gap was constructed by PCR amplification of a Rap1Gap cDNA, obtained from Open Biosystems, and this product was then subcloned into the *Bam* HI and *Eco* RV sites of the previously described pcDNA3-FLAG vector (12). Constructs encoding the amino acids 1 to 1,530 and 1,531 to 1,807 of TSC2 were cloned by amplifying the relevant sequences of a previously described pcDNA3-FLAG-TSC2 construct (12) and ligating them into the *Bam* HI and *Xba* I sites of pcDNA3-FLAG. Similarly, cDNAs encoding amino acids 1 to 1,128 and 1,129 to 1,708 of Rictor were cloned by PCR-amplifying the relevant sequences from a pRK5-myc-Rictor cDNA. All other myc-tagged mTORC2 components were obtained from Addgene and/or described elsewhere (5, 10).

Cell lysates were prepared from near-confluent 100 mm dishes of HEK293 cells in lysis buffer derived from previous studies on the mTOR complexes (9), and immunoprecipitations were done as described elsewhere (10). Antibodies to Rictor and mSin1 were obtained from Bethyl Laboratories, phospho-PKCα (S657) from Upstate, NDRG1 from Abcam, the FLAG epitope and actin from Sigma, and preimmune rabbit IgG from Santa Cruz Biotechnology. All other antibodies were obtained from Cell Signaling Technology.

mTORC2 kinase assays. Kinase assays on endogenous mTORC2 were done as described previously (10). Where indicated, purified FLAG-tagged TSC1-TSC2 complexes or Rap1Gap were added at the start of the kinase reactions. To purify these FLAG-tagged proteins, HEK293 cells were transfected with low concentrations of the given construct (2 μg/15 cm plate) and lysed with NP-40 lysis buffer [20 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 1 mmol/L $MgCl_2$, 1% NP-40, 10% glycerol, 50 mmol/L glycerol 2-phosphate, and 50 mmol/L NaF]. Proteins were immunoprecipitated with anti-FLAG (M2) affinity gel and washed in the above lysis buffer containing 500 mmol/L NaCl. The FLAG-tagged proteins were eluted with 100 μg/mL 3xFLAG peptide (Sigma) diluted in kinase reaction buffer. The concentration of the purified protein was determined by silver stain following SDS-PAGE using bovine serum albumin standards.

Immunohistochemistry. The *Tsc2*$^{+/-}$ mice used in this study were described previously (13, 14). For immunohistochemistry, serial 4 μm sections were cut from formalin-fixed and paraffin-embedded kidneys from 1-year-old *Tsc2*$^{+/-}$ mice. The sections were autoclaved in 10 mmol/L citrate buffer (pH 6.0) for 10 min for antigen retrieval followed by immersion in 3% hydrogen peroxide for 10 min and 4% normal goat serum for 1 h to block endogenous peroxidase and nonspecific antibody binding, respectively. After incubation with the appropriate primary antibodies overnight at 4°C [total PKCα at 1:50 dilution and phospho-S6 (S240/244) at 1:100 dilution], secondary antibodies and avidin-biotin-peroxidase complex were applied according to the manufacturer's protocol (Vectastain). Visualization was achieved by incubating with 3,3′-diaminobenzidine tetrachloride (Pierce), and the sections were counterstained with hematoxylin.

Formalin-fixed, paraffin-embedded sections of a TSC patient-associated renal angiomyolipoma were obtained from Massachusetts General Hospital. These studies were approved by the Human Study Committee. Histopathologic diagnosis of angiomyolipoma was confirmed by two independent pathologists. The antibodies used above, as well as those to phospho-Akt (S473; 1:50 dilution), were applied to consecutive 5 μm tissue sections prepared for immunohistochemical analysis, as described above. Immunodetection was done with a LSAB 2 system (DAKO). Hematoxylin was used as a counterstain for the PKCα staining.

Results

Loss of TSC2 decreases the phosphorylation of both PI3K-dependent and PI3K-independent substrates of mTORC2. Because mTORC2 has been implicated in the phosphorylation of hydrophobic motifs on several AGC family kinases (4–6), we examined the phosphorylation of these motifs in cells lacking either the TSC1-TSC2 complex (*Tsc2*$^{-/-}$ MEFs) or mTORC2 (*Rictor*$^{-/-}$ MEFs) using a phospho-specific antibody that recognizes the phosphorylated hydrophobic motif. Immunoblots with this antibody detected just two prominent bands in the wild-type MEFs, a protein of ~60 kDa that is insulin responsive and acutely sensitive to the PI3K inhibitor wortmannin and a protein of ~75 kDa that is constitutively phosphorylated and wortmannin insensitive (Fig. 1*A*). Interestingly, the phosphorylation of both of these proteins is severely blunted in *Tsc2*$^{-/-}$ MEFs and is undetectable in *Rictor*$^{-/-}$ MEFs. Based on their regulation and molecular mass, it is likely that these 60 and 75 kDa proteins are Akt and PKCα, respectively. Phosphorylation of the hydrophobic motif on PKCα (S657) is mediated by mTORC2 in a manner independent of PI3K signaling (4, 5, 7, 8) and is required for PKCα stability (reviewed in ref. 15). Indeed, we find that PKCα-S657 phosphorylation and total protein levels are reduced in both knockout lines relative to their littermate-derived wild-type counterparts (Fig. 1*B, i*). Importantly, these effects on PKCα can be rescued by stably reconstituting the *Tsc2*$^{-/-}$ MEFs with human *TSC2* but not empty vector (Fig. 1*B, ii*).

The reduction in PKCα phosphorylation and levels in *Tsc2*$^{-/-}$ cells is obvious in both serum-starved and serum-fed cells (Fig. 1*C*). The strong decrease in Akt hydrophobic motif (S473) phosphorylation in cells lacking the TSC1-TSC2 complex can be attributed to a combination of mTORC1-dependent feedback mechanisms (reviewed in ref. 16) and loss of mTORC2 activity (10). To examine whether the elevated mTORC1 signaling in *Tsc2*$^{-/-}$ cells contributes to the attenuation of PKCα, we tested the effects of prolonged rapamycin treatment. Strikingly, overnight exposure to rapamycin further decreased PKCα phosphorylation in *Tsc2*$^{-/-}$ MEFs and slightly inhibited this phosphorylation in wild-type cells (Fig. 1*C*). However, this same treatment increases Akt-S473 phosphorylation in both cell lines. As reported previously in other cells (17), prolonged rapamycin disrupts the mTORC2 complex, as detected by a large decrease in the amount of mTOR coimmunoprecipitating with Rictor, in both wild-type and *Tsc2*$^{-/-}$ MEFs (Fig. 1*D*). Therefore, unlike Akt phosphorylation, which is also affected by feedback regulation from mTORC1, the decrease in PKCα phosphorylation is the specific result of loss of mTORC2 activity in *Tsc2*$^{-/-}$ cells.

A second conserved motif on Akt and PKCα, called the turn motif (T450 on Akt1), has also been found to be phosphorylated in a manner dependent on mTORC2 activity (7, 8). Like the hydrophobic motif on PKCα, the turn motif on these kinases is phosphorylated constitutively as part of protein folding and maturation (reviewed in ref. 15). Although not as diminished as in the *Rictor*$^{-/-}$ MEFs, the phosphorylation of Akt-T450 is reproducibly lower in *Tsc2*$^{-/-}$ MEFs relative to their wild-type counterparts (Fig. 1*B, i*), and this can be restored by reconstitution with TSC2 (Fig. 1*B, ii*). Collectively, these data show that the TSC1-TSC2 complex promotes both PI3K-dependent functions of mTORC2 (Akt-S473 phosphorylation) and basal constitutive activities of mTORC2 (PKC-S657 and Akt-T450 phosphorylation).

PKCα levels are decreased in mouse and human tumors lacking a functional TSC1-TSC2 complex. The TSC disease is

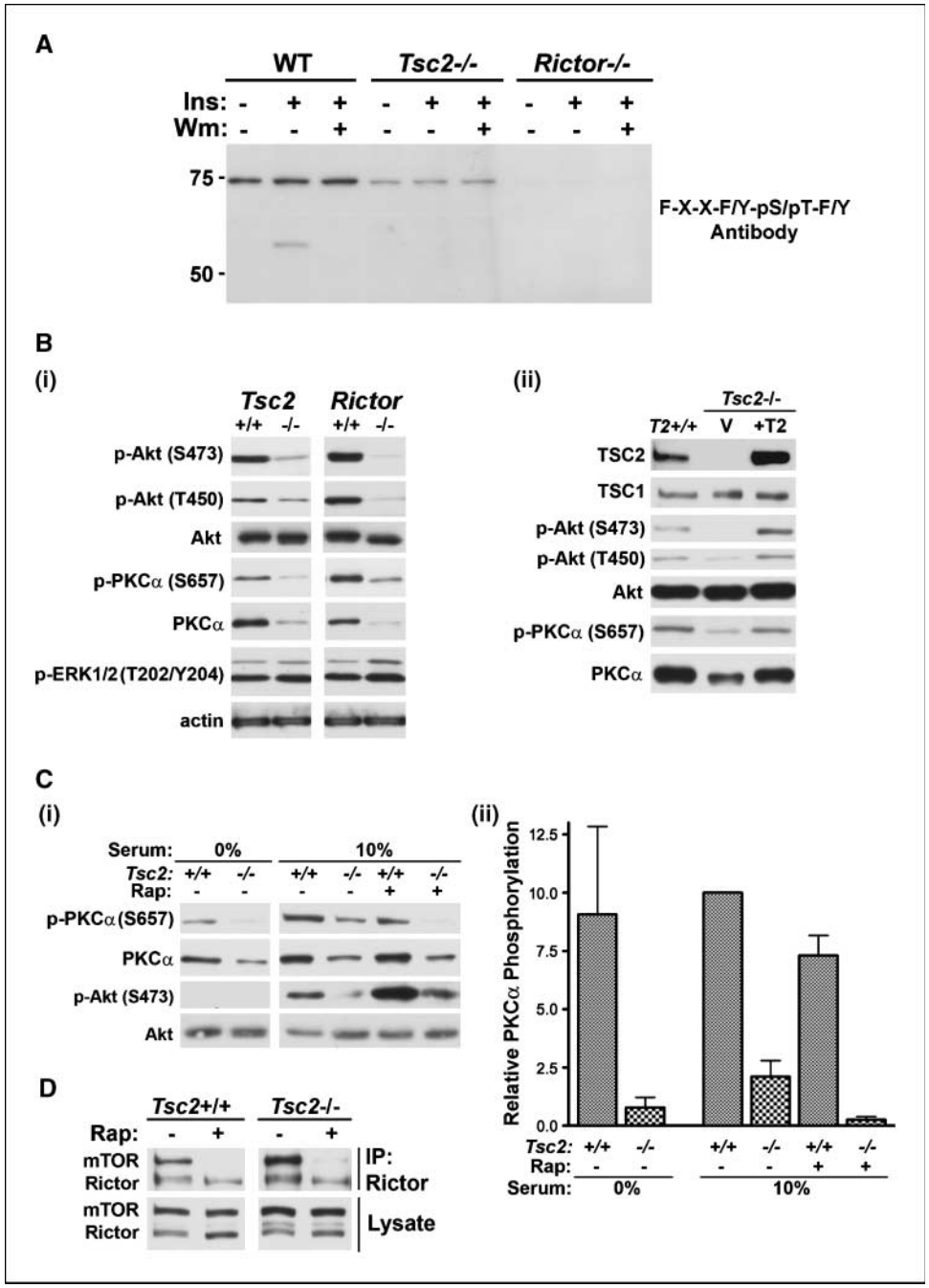

Figure 1. Phosphorylation of mTORC2 substrates is attenuated in *Tsc2*-null cells. *A, Tsc2*$^{+/+}$ (*WT*), *Tsc2*$^{-/-}$, and *Rictor*$^{-/-}$ MEFs were serum starved overnight and stimulated with insulin (100 nmol/L) for 15 min with or without 15 min pretreatment with wortmannin (100 nmol/L). An immunoblot using the phospho-PDK1-docking motif antibody, which recognizes phosphorylated hydrophobic motifs on AGC kinases. *B, i,* littermate-derived pairs of wild-type and either *Tsc2*$^{-/-}$ or *Rictor*$^{-/-}$ MEFs were cultured in 10% serum before lysis; *ii,* wild-type MEFs were compared with *Tsc2*$^{-/-}$ MEFs reconstituted with empty vector (*V*) or human *TSC2* (*+T2*). *C, i, Tsc2*$^{+/+}$ and *Tsc2*$^{-/-}$ MEFs were serum starved or cultured in 10% serum for 17 h in the presence or absence of rapamycin (20 nmol/L); *ii,* experiment described in *i* repeated three independent times, quantified using ImageJ software, and normalized to levels in *Tsc2*$^{+/+}$ cells. Mean ± SE. *D,* MEFs were treated as in *C*. Levels of mTOR and Rictor in Rictor immunoprecipitates and lysates.

caused by germ-line mutations in one copy of either *TSC1* or *TSC2* gene, with the numerous individual tumors generally arising due to somatic "second-hit" mutations or loss of heterozygozity (1). Similarly, rodent models of TSC that are heterozygous for *Tsc1* or *Tsc2* develop tumors through loss of heterozygozity, and the resulting tumors in both humans and rodents display elevated mTORC1 signaling. To determine whether there is a reciprocal decrease in mTORC2 signaling in these tumors, we examined PKCα levels in kidney tumors from *Tsc2*$^{+/-}$ mice and TSC patients, as total levels of PKCα reflect its phosphorylation by mTORC2. In kidneys from *Tsc2*$^{+/-}$ mice, the majority of normal renal tubules, and all glomeruli, stain strongly for PKCα (Fig. 2*A* and *B*). However, the cells comprising all three of the highly penetrant tumor types arising in these kidneys (cysts, cystadenomas, and solid adenomas; ref. 13) are nearly devoid of PKCα staining. This is in contrast to phosphorylation of S6 downstream of mTORC1, which is elevated in serial sections of these tumors (Fig. 2*C*).

Renal angiomyolipomas are among the most common tumors in TSC patients. These are unusual highly vascular tumors composed of aberrant thick-walled blood vessels, smooth muscle-like cells, and adipocytes, all of which display TSC gene loss of heterozygozity (18, 19). Relative to adjacent normal renal parenchyma, PKCα protein levels are not detectable in either the smooth muscle or thick-walled vessels of angiomyolipomas from TSC patients (Fig. 3*A*). Consistent with the expected increase in mTORC1 signaling, TSC-associated angiomyolipomas exhibit increased

phospho-S6 levels (Fig. 3*B*). However, Akt-S473, a second mTORC2 target, is reduced in its phosphorylation in these tumors. Therefore, in contrast to mTORC1, events downstream of mTORC2 are attenuated in tumors lacking a functional TSC1-TSC2 complex.

Loss of TSC2 leads to defects in SGK1 signaling. It was recently shown that phosphorylation of the hydrophobic motif on SGK1 (S422), which is essential for its activation, is also mediated by mTORC2 (6). Because the amino acid sequence surrounding S422 on SGK1 is nearly identical to the hydrophobic motifs of S6K1 and other AGC kinases, available phospho-specific antibodies to this site are limited in both their specificity and sensitivity (6). Although some downstream targets of SGK1 can also be phosphorylated by Akt, T346 on the NDRG1 protein is strictly phosphorylated by SGK1 (20) and serves as a very specific readout of both mTORC2 and SGK1 activity (6). As in *Rictor*$^{-/-}$ MEFs, loss of TSC2 expression in the *Tsc2*$^{-/-}$ MEFs leads to a dramatic reduction in the phosphorylation of NDRG1 (Fig. 4*A*). Whereas there are slight differences between *Tsc2*$^{+/+}$ and *Tsc2*$^{-/-}$ cells, SGK1 protein levels are elevated in the *Rictor*$^{-/-}$ cells relative to the *Rictor*$^{+/+}$ cells. The nature of this increase is unknown. Importantly, the phosphorylation of NDRG1 was restored in *Tsc2*$^{-/-}$ MEFs stably reconstituted with wild-type *TSC2* but not empty vector (Fig. 4*B*). Therefore, like other mTORC2 targets (Akt and PKCα), SGK1 activation is defective on loss of the TSC1-TSC2 complex.

TSC1-TSC2 complex can stimulate the *in vitro* kinase activity of mTORC2. In a previous study, we found that the TSC1-TSC2 complex can physically associate with mTORC2, but not mTORC1, and that the mTORC2 associated with the TSC1-TSC2 complex was catalytically active (10). To determine if the TSC1-TSC2 complex can directly stimulate mTORC2, we tested whether purified TSC1-TSC2 complex could increase the *in vitro* kinase activity of mTORC2 isolated from *Tsc2*$^{-/-}$ MEFs. To purify properly folded TSC1-TSC2 complex, FLAG-tagged TSC1 and TSC2 were isolated from HEK293 cells, which we found in previous studies yields an active complex with robust GAP activity toward Rheb (21). To avoid copurification of endogenous mTORC2, we washed the immunoprecipitates with a high-salt wash buffer found previously to disrupt binding of mTORC2 to the TSC1-TSC2 complex (10). Subsequent elution of the immunoprecipitates yielded a rather pure stoichiometric complex of TSC1 and TSC2 (Fig. 5*A*), which was added in increasing quantities (0-10 units) to Rictor immunoprecipitates from *Tsc2*$^{-/-}$ MEFs. Interestingly, the very low kinase activity of mTORC2 isolated from insulin-stimulated *Tsc2*-null cells was increased on addition of purified TSC1-TSC2 complex (Fig. 5*B*). Consistent with our previous overexpression studies (10), we found that the addition of purified TSC1-TSC2 complexes can also further stimulate mTORC2 activity from *Tsc2*$^{+/+}$ MEFs (Fig. 5*C*). It is worth noting that, whereas

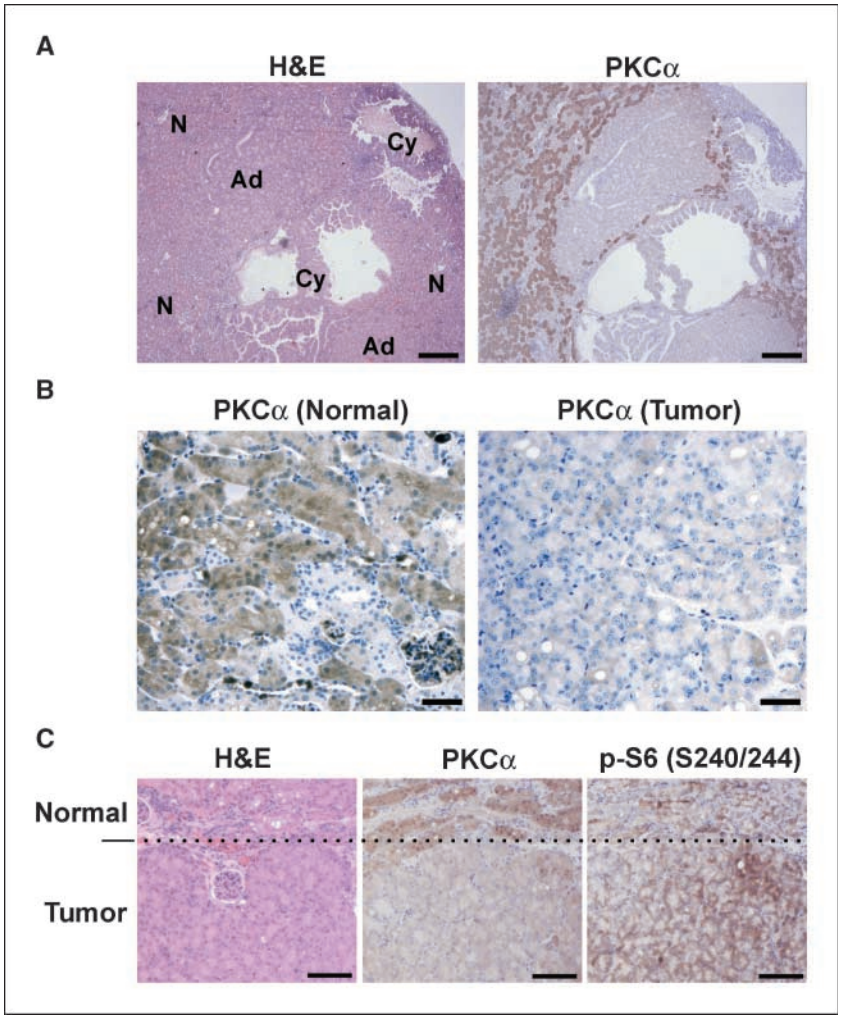

Figure 2. PKCα levels are reduced in kidney tumors from *Tsc2*$^{+/-}$ mice. *A,* serial sections of a kidney from a *Tsc2*$^{+/-}$ mouse (age 1 year) stained with H&E (*left*) or PKCα antibodies (*right*) showing a relative decrease in PKCα levels in the cystic adenomas (*Cy*) and solid adenomas (*Ad*) relative to the normal renal parenchyma (*N*). Magnification, ×40. *Bar*, 250 μm. *B,* representative images of normal renal tubular epithelium (*left*) and adenoma (*right*) from a *Tsc2*$^{+/-}$ mouse kidney stained with PKCα antibodies. Magnification, ×200. *Bar*, 50 μm. *C,* decreased PKCα levels and increased phospho-S6 levels in an adenoma compared with adjacent normal renal parenchyma from a *Tsc2*$^{+/-}$ mouse kidney. Representative images of the same region of a kidney tumor stained with H&E (*left*), PKCα antibodies (*middle*), or phospho-S6 (S240/244; *right*). Magnification, ×100. *Bar*, 100 μm.

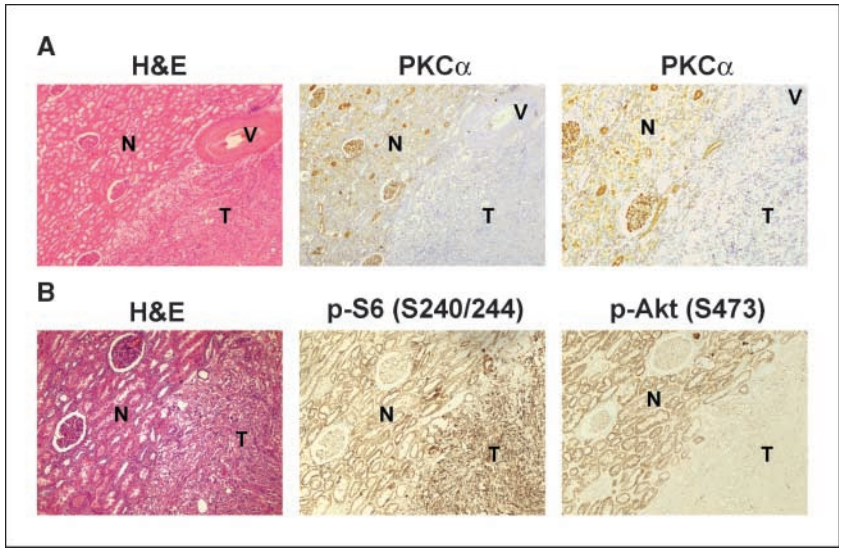

Figure 3. PKCα levels are reduced in TSC-associated kidney angiomyolipoma. *A,* serial sections of a kidney from a TSC patient were stained with H&E (*left*; magnification, ×25) or PKCα antibodies [magnification, ×25 (*middle*) and ×50 (*right*)] and counterstained with hematoxylin (*blue*). *N,* normal renal parenchyma; *T,* smooth muscle-like component of tumor; *V,* tumor-associated blood vessel. *B,* decreased phospho-Akt (S473) and increased phospho-S6 (S240/244) levels in an angiomyolipoma compared with adjacent normal renal parenchyma from a TSC patient shown in serial sections (magnification, all ×100).

addition of the TSC1-TSC2 complex results in a 2-fold increase in the activity of mTORC2 isolated from both cell lines, the level of activity from wild-type cells remains ~10-fold higher than in *Tsc2*$^{-/-}$ cells. This is consistent with the *Tsc2*-null cells being unresponsive to insulin due to mTORC1-dependent feedback mechanisms. To show the specificity of this effect for the TSC1-TSC2 complex, we similarly purified FLAG-tagged Rap1Gap, which is among the most highly homologous proteins to TSC2, and compared its effects on mTORC2 activity with that of the same concentration of purified FLAG-TSC2. In contrast to the stimulation by TSC2, addition of Rap1Gap had no activating effect on mTORC2 immunoprecipitated from *Tsc2*$^{-/-}$ cells (Fig. 5*D*). Therefore, it appears that the TSC1-TSC2 complex has direct effects on mTORC2 kinase activity, the molecular nature of which is currently unknown.

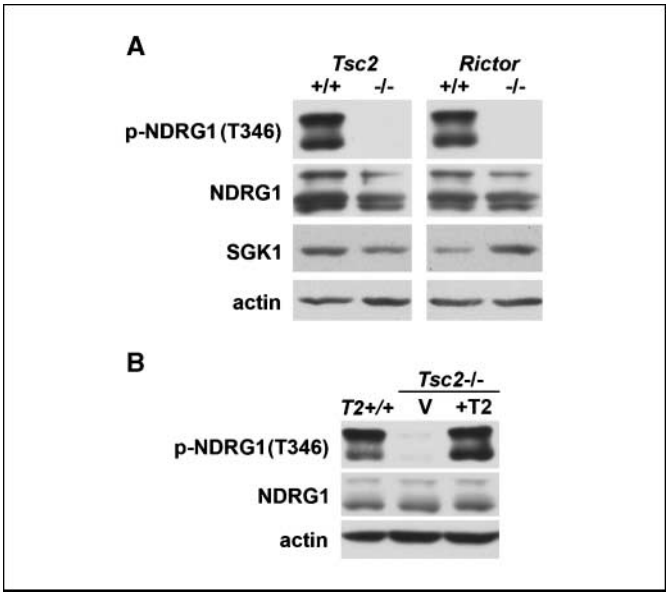

Figure 4. Loss of SGK1 activity in *Tsc2*-deficient cells. *A,* littermate-derived pairs of wild-type and either *Tsc2*$^{-/-}$ or *Rictor*$^{-/-}$ MEFs were cultured in 10% serum before lysis. *B,* littermate-derived wild-type MEFs were compared with *Tsc2*$^{-/-}$ MEFs reconstituted with empty vector or human *TSC2*.

NH_2-terminal region of TSC2 and COOH-terminal region of Rictor mediate the interaction between the TSC1-TSC2 complex and mTORC2. Within the TSC1-TSC2 complex, TSC2 was found previously to be essential for the association of the complex with mTORC2 (10). We find that endogenous mTOR, Rictor, and TSC1 can coimmunoprecipitate with either full-length TSC2 or a truncated mutant of TSC2 (TSC2-N, amino acids 1-1,530) lacking its GAP domain, whereas none of these proteins coprecipitate with the GAP domain alone (amino acids 1,531-1,807; Fig. 6*A*). Therefore, consistent with the GAP activity of TSC2 not being required for the effects of the TSC1-TSC2 complex on mTORC2 (10), TSC2 associates with mTORC2 components in a manner independent of its GAP domain.

The interaction between mTORC2 components (mTOR, Rictor, mSIN1, and mLST8) is highly complex, and to date, the precise interaction domains between each of the components have not been determined. In *Saccharomyces cerevisiae*, the presence of each individual component of TORC2 is essential for associations between the other components (22). To identify the critical subunit of mTORC2 that mediates the interaction with the TSC1-TSC2 complex, we co-overexpressed the core components of mTORC2 and examined the ability of these proteins to coimmunoprecipitate with the TSC1-TSC2 complex using FLAG-TSC1 as a handle. All components of mTORC2 coimmunoprecipitated with the TSC1-TSC2 complex (Fig. 6*B*). However, when either mTOR or mSin1 was omitted, only Rictor bound to the TSC1-TSC2 complex, showing that Rictor is sufficient for the association. Reciprocally, omission of Rictor alone resulted in loss of all components of mTORC2 from the TSC1-TSC2 complex immunoprecipitate. These results indicate that Rictor is the essential component of mTORC2 for its physical association with the TSC1-TSC2 complex.

The strongest amino acid sequence conservation between Avo3p (the *S. cerevisiae* orthologue of Rictor) and Rictor from either *Drosophila* or human lies within a large NH_2-terminal region (Fig. 6*C, dark gray*), with the COOH-terminal region of Avo3p possessing a smaller, more weakly conserved domain (Fig. 6*C, lighter gray*). Full-length Rictor was able to coimmunoprecipitate endogenous mTOR, mSin1, and TSC2 (Fig. 6*C*). Somewhat surprisingly, neither the NH_2-terminal nor the COOH-terminal

halves of Rictor were sufficient to associate with mTOR or mSIN1. However, whereas the NH_2-terminal fragment was impaired in its interaction with TSC2, the COOH-terminal fragment bound more strongly to TSC2 than full-length Rictor. These data further show that Rictor can associate with TSC2 in a manner independent of other mTORC2 components and suggest that the COOH-terminal region of Rictor mediates this interaction.

Discussion

It is well established that the TSC1-TSC2 complex is a critical negative regulator of mTORC1 signaling that can sense cellular growth conditions through a large number of upstream pathways (reviewed in ref. 2). *TSC* gene mutations, therefore, give rise to cells and tumors with elevated and uncontrolled mTORC1 activation. Combined with our previous study (10), our results here identify mTORC2 activation as a second distinct molecular function of the TSC1-TSC2 complex. We show that impaired mTORC2 signaling accompanies the aberrant activation of mTORC1 as a major molecular defect triggered by TSC gene disruption, and it is likely that both contribute to the multifaceted pathologic properties of the TSC and lymphangioleiomyomatosis diseases.

Multiple feedback mechanisms stemming from elevated mTORC1 signaling are active in cells lacking the TSC1-TSC2 complex (23–25), and these contribute to the loss of growth factor–stimulated Akt phosphorylation. However, our previous study showed that the loss of mTORC2 activity can be separated from the elevated mTORC1 signaling in these cells (10). Our finding here that the regulation of growth factor–independent targets of mTORC2 is also defective in *Tsc2*$^{-/-}$ cells supports the conclusion that mTORC2 activity is impaired in these cells through mechanisms other than, or in addition to, mTORC1-dependent feedback inhibition of growth factor and PI3K signaling. It is worth noting that the loss of phosphorylation of mTORC2 substrates in *Tsc2*$^{-/-}$ MEFs is not as complete as in their *Rictor*$^{-/-}$ counterparts. This is consistent with our previous conclusion that the TSC1-TSC2 complex is a regulator of mTORC2 rather than a core component of the complex itself. Finally, our ability to partially reconstitute the stimulation of mTORC2 kinase activity with purified TSC1-TSC2 complex *in vitro* shows that this regulation is direct rather than through secondary signaling events. Future studies are clearly needed to delineate the precise molecular mechanism of this regulation, but our studies strongly suggest the involvement of direct physical interactions between these two complexes.

Aberrant mTORC1 activation is a common molecular event in many human cancers, and activation of mTORC1 has been found to be required for neoplastic transformation by several oncogenes, such Ras, PI3K, and Akt (e.g., refs. 26–28). It is striking that loss of the TSC tumor suppressors, which gives rise to very high levels of constitutive mTORC1 signaling, results in a tumor syndrome characterized largely by benign growths (1), indicating that elevated mTORC1 activity alone is not sufficient for the

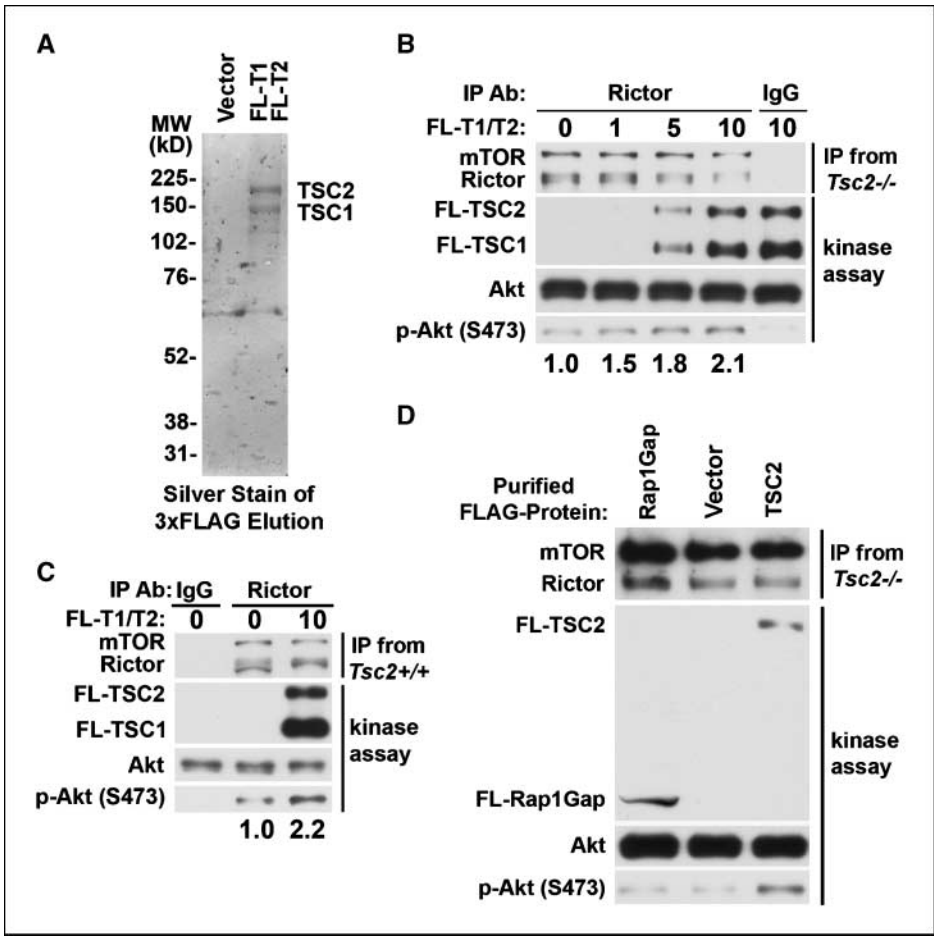

Figure 5. Purified TSC1-TSC2 complex increases the *in vitro* kinase activity of mTORC2. *A,* TSC1-TSC2 complex was purified as detailed in Materials and Methods. The eluate was separated by SDS-PAGE and visualized by silver staining. *B,* lysates from insulin-stimulated (100 nmol/L for 15 min) *Tsc2*$^{-/-}$ MEFs were subjected to immunoprecipitation with either Rictor antibodies or control IgG. Rictor immunoprecipitate was divided into equal fractions for separate kinase reactions using Akt1 purified from insect cells as the substrate. Increasing quantities of purified FLAG-TSC1-TSC2 complex were added to each kinase reaction (1 unit contains 1-2 ng of purified complex). mTORC2 kinase activity was assessed by Akt-S473 phosphorylation, and the activity relative to the 0 unit control is indicated. *C,* effects of the TSC1-TSC2 complex on the kinase activity of mTORC2 from insulin-stimulated *Tsc2*$^{+/+}$ MEFs was assessed as in *B. D,* effects of equal concentrations of FLAG eluates containing either FLAG-Rap1Gap or FLAG-TSC2 on the kinase activity of mTORC2 immunoprecipitated from *Tsc2*$^{-/-}$ MEFs were assessed as in *B*.

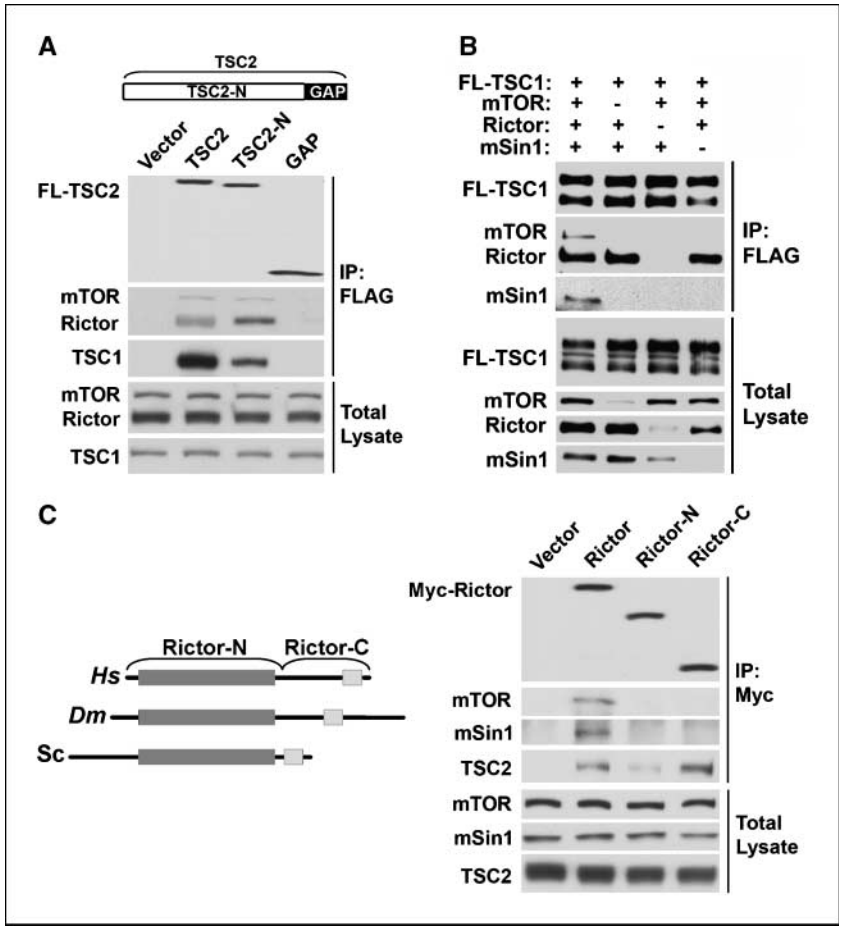

Figure 6. NH_2-terminal region of TSC2 and COOH-terminal region of Rictor mediate the interaction between the TSC1-TSC2 complex and mTORC2. *A,* HEK293 cells were transfected with empty vector, FLAG-TSC2 (amino acids 1-1,807), FLAG-TSC2-N (amino acids 1-1,530), or FLAG-TSC2-GAP (amino acids 1,531-1,807). The presence of endogenous mTOR, Rictor, and TSC1 was then assessed in anti-FLAG immunoprecipitates. *B,* HEK293 cells were cotransfected with FLAG-TSC1, myc-mLST8, and the other indicated mTORC2 components. The presence of the various exogenously expressed mTORC2 components was then assessed in anti-FLAG immunoprecipitates. *C, left,* conserved regions of Rictor from human (*Hs*), *Drosophila* (*Dm*), and *S. cerevisiae* (*Sc*). The NH_2-terminal region (*dark gray*) is much more highly conserved than the small region of homology at the COOH terminal (*light gray*). *Right,* HEK293 cells were transfected with empty vector, myc-Rictor (amino acids 1-1,708), myc-Rictor-N (amino acids 1-1,128), or myc-Rictor-C (amino acids 1,129-1,708). The presence of endogenous mTOR, mSin1, and TSC2 was then assessed in anti-myc immunoprecipitates.

development of malignancies. Interestingly, recent studies have found that mTORC2 activity is important for malignant transformation in some settings (29–31). It is currently unclear whether the role of mTORC2 in cancer is mediated solely through Akt or also through its other downstream targets, PKCα and SGK1. Classic PKCs, such as PKCα, are potently activated by tumor-promoting phorbol esters and have been shown to promote tumor cell viability, proliferation, and invasion (e.g., refs. 32–34). Furthermore, PKCα protein levels have been reported to be elevated in carcinomas (e.g., refs. 33, 35). This is in stark contrast to our observations in the benign kidney tumors of $Tsc2^{+/-}$ mice and TSC patients, which have greatly reduced levels of PKCα. It is worth noting that, in addition to regulation by mTORC2, PKCα phosphorylation and stability are affected by its activation state, as it is dephosphorylated and degraded more rapidly once active (36–38). Because mTORC2 activity is greatly diminished in TSC gene-deficient cells (10), the reduced PKCα levels detected in these tumors are likely the result of reduced mTORC2 activity rather than activation-induced dephosphorylation. Therefore, in addition to mTORC1-dependent feedback mechanisms affecting PI3K activation, which we have shown previously to contribute to the slow growth of tumors lacking the *TSC* genes (14), our current study suggests that loss of mTORC2 activity will also contribute to the benign nature of *TSC*-deficient tumors.

These observations suggest a very unusual role for the *TSC* genes as tumor suppressors. The TSC1-TSC2 complex might play a dual role, both inhibiting and promoting tumor formation through mTORC1 inhibition and mTORC2 activation, respectively. In support of this idea, a survey of available gene expression array data sets of human cancer samples (via the Oncomine database;[3] ref. 39) reveals that *TSC2* is significantly up-regulated in solid tumors of the brain, lung, and prostate and is down-regulated only in hematologic malignancies (40). We speculate that the TSC1-TSC2 complex might act to promote tumorigenesis in some cell types due to its effect on mTORC2, especially when mTORC1 is not needed for malignant transformation or is activated through pathways independent of this complex.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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[3] http://www.oncomine.org

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