

cAMP-Dependent Cytosolic Mislocalization of p27^{kip}-Cyclin D1 During Quinol-Thioether-Induced Tuberous Sclerosis Renal Cell Carcinoma

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The loss of tuberin, the tuberous sclerosis-2 (*Tsc-2*) gene product, is associated with cytoplasmic mislocalization of p27 in uterine leiomyomas derived from Eker rats (*Tsc-2*^{EK/+}) and in human metastatic renal cell carcinoma tissue. Signaling associated with cytoplasmic mislocalization of p27 in renal cancer is relatively unknown. Renal tumors derived from 2,3,5-tris-(glutathion-S-yl)hydroquinone (TGHQ)-treated *Tsc-2*^{EK/+} rats, and null for tuberin, display elevated nuclear and cytosolic p27, with parallel increases in cytosolic cyclin D1 levels. Similar changes are observed in TGHQ-transformed renal epithelial cells derived from *Tsc-2*^{EK/+} rats (QTRRE cells), which, in addition to the cytoplasmic mislocalization of p27 and cyclin D1, exhibit high ERK, B-Raf, and Raf-1 kinase activity. Renal tumor xenografts, derived from subcutaneous injection of QTRRE cells into nude mice, also display increases in cytosolic mislocalization of p27 and cyclin D1. Dibutyryl cAMP and/or phosphodiesterase inhibitors (PIs; pentoxifylline or theophylline) increase Rap1B activation, B-Raf kinase activity, and cytosolic p27/cyclin D1 protein levels in QTRRE cells. Inhibition of Raf kinases with either sorafenib or B-Raf small interfering RNA (siRNA) caused a mitogen-activated protein kinase-mediated downregulation of p27. Moreover, decreases in cyclin D1 were also associated with p27 siRNA knockdown in QTRRE cells. Finally, theophylline-mediated increases in p27 and cyclin D1 were attenuated by sorafenib, which modulated Raf/MEK/ERK signaling. Collectively, these data suggest that the cAMP/Rap1B/B-Raf pathway modulates the expression of p27 and the cytoplasmic mislocalization of p27-cyclin D1 in tuberous sclerosis gene-regulated-renal cancer. Therefore, the loss of tuberin and engagement of the cAMP pathway may independently direct p27-cyclin D1 cytosolic stabilization during renal tumor formation.

Key Words: B-raf; p27; cAMP; cyclin D1; renal cell carcinoma; quinol-thioether.

Tuberous sclerosis complex (TSC) is an autosomal dominant hereditary disease, with a high rate of spontaneous mutations in *Tsc-1* and *Tsc-2* genes (Kida *et al.*, 2005). Mutations in TSC genes occur during human renal tumor formation (Bernstein and Robbins, 1991; Bjornsson *et al.*, 1996), and loss of heterozygosity

(LOH) at the *Tsc-2* locus occurs in renal tumors of TSC and sporadic renal cell carcinoma (RCC) patients (Carbonara *et al.*, 1996; Henske *et al.*, 1995). The tumor suppressor tuberin, the protein product of *Tsc-2*, can affect subcellular localization and protein accumulation of the cyclin-dependent kinase inhibitor p27 (kip) (Rosner and Hengstschlager, 2004; Short *et al.*, 2008). Tuberin also complexes with p27 to promote its nuclear localization (Rosner *et al.*, 2007) and inhibit its ubiquitin-mediated degradation by Skp1/cullin/F-box (SCF) proteasome (Rosner *et al.*, 2008; Rosner and Hengstschlager, 2004).

Nuclear p27 typically associates with distinct cyclin-dependent kinase (CDK)-cyclin complexes to initiate cell cycle arrest. Complex interactions between CDKs, cyclins, and p27 govern the G1-S phase transition of the cell cycle. During the G1 checkpoint, D-type cyclins (D1, D2, and D3) bind and activate CDK4/6 (Malumbres and Barbacid, 2009). Due to the instability of the cyclin D1-CDK4 complex, p27 interaction is necessary for complex assembly and stabilization (Cheng *et al.*, 1999; Sherr and Roberts, 1999). Thus, p27 is an extremely versatile CDK inhibitor, in that it can regulate nuclear cell cycle exit and proliferation (Blain, 2008; Vervoorts and Luscher, 2008). Nuclear p27 is typically characterized as a tumor suppressor, but recent developments have revealed the duality of p27 as an oncogene in certain cancers, subsequent to its cytoplasmic mislocalization.

In human kidney, breast, colon, ovarian, thyroid, and esophageal cancers, cytoplasmic mislocalization of p27 correlates with an aggressive tumor type and poor prognosis (Alkarain *et al.*, 2004; Ciaparrone *et al.*, 1998; Hennenlotter *et al.*, 2008; Masciullo *et al.*, 2000; Motti *et al.*, 2005; Pantuck *et al.*, 2007; Singh *et al.*, 1998; Viglietto *et al.*, 2002). Analysis of *Tsc2* null uterine leiomyomas from the Eker rat (*Tsc-2*^{EK/+}), *Tsc2*^{-/-} mouse embryonic fibroblasts (MEFs), and microscopic kidney lesions in *Tsc2*^{+/-}/p27^{+/+} mice and *Tsc2*^{+/-}/p27^{+/-} mice, all revealed cytoplasmic mislocalization of p27 (Short *et al.*, 2008). The molecular mechanisms facilitating the cytoplasmic retention of p27, and its ability to evade targeted degradation, remain complex.

Degradation of p27 is necessary for the G₁ to S phase transition to occur and is initiated by phosphorylation of p27 by cyclin E- and A-CDK2, translocating p27 to the cytoplasm for SCF E3 ubiquitin ligation (Carrano *et al.*, 1999; Sheaff *et al.*, 1997; Sutterluty *et al.*, 1999; Vlach *et al.*, 1997). Binding of p27 to cyclin D-CDK4 complexes protects p27 from degradation by the SCF^{SKP1} E3-ubiquitin-ligase complex (Kamura *et al.*, 2004). As p27^{Kip1} has never been found unbound *in vivo* (Vervoorts and Luscher, 2008) and cytoplasmic p27-cyclin D-CDK4 complexes have intact kinase activity (Blain *et al.*, 1997; James *et al.*, 2008), an oncogenic function of p27 may reside in its maintenance of constitutive p27-cyclin D-CDK4 activity. Cyclin D1 is overexpressed in a variety of cancers (Kim and Diehl, 2009). Moreover, in tuberin null renal tumors and renal cells derived from *Tsc-2*^{EK/+} rats, the loss of tuberin expression is associated with an increase in cyclin D1 protein levels (Soucek *et al.*, 1997; Yoon *et al.*, 2002) and with an increase in cyclin D1 gene expression (Patel *et al.*, 2003).

The signaling driving p27 expression, localization, and stabilization in RCC is relatively unknown. In a variety of cell types, the cyclic adenosine monophosphate (cAMP) pathway is associated with increased levels of p27 (Alderson and Hama, 2009; Bond *et al.*, 2008; da Silva *et al.*, 2008; Paris *et al.*, 2006). In retinal, schwannoma, and neuronal cells, the cAMP mediated increase in p27 is also linked to cell cycle exit and differentiation (Alderson and Hama, 2009; da Silva *et al.*, 2008; Paris *et al.*, 2006). Increases in cAMP also mediate activation of Rap1 GTPase-activating protein 1 (Rap1) and subsequent downstream activation of B-Raf (Garcia *et al.*, 2001; Grewal *et al.*, 2000; Vossler *et al.*, 1997). Rap activation of ERK is dependent on the expression levels of B-Raf. In cell lines lacking B-Raf expression, Rap1 inhibits Raf-1/ERK activation (Dugan *et al.*, 1999; Okada *et al.*, 1998; Schmitt and Stork, 2001). These data suggest that cAMP signaling can regulate p27 expression and Rap-GTP/B-Raf activation of the mitogen-activated protein kinase (MAPK) cascade.

In the present study, we examined the relationship between cAMP, the B-Raf/ERK MAPK cascade, and p27 in tuberin-deficient renal tumor models: 2,3,5-tris-(glutathion-S-yl)hydroquinone (TGHQ)-induced renal tumors in *Tsc-2*^{EK/+} rats, TGHQ-transformed *Tsc-2*^{EK/+} cells (QTRRE), and QTRRE renal tumor xenografts in nude mice. Our data identify cAMP as a modulator of Rap/B-Raf activity, B-Raf MAPK expression of p27, and p27-cyclin D1 cytosolic mislocalization in tuberous sclerosis renal carcinogenesis.

MATERIALS AND METHODS

Cell cultures. Tuberin-negative quinol-thioether-transformed rat renal epithelial (QTRRE-2, QTRRE-3) cells were established from primary renal epithelial cells (Yoon *et al.*, 2001). Human kidney cells (HK2) and LLC-PK1 (porcine proximal tubule epithelial cells) were obtained from the American Type Culture Collection (Manassas, VA). HK2 and QTRRE cells were grown in Dulbecco's modified Eagle's medium (DMEM)/F12 (1:1) (Invitrogen,

Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS). LLC-PK1 cells were maintained in DMEM supplemented with 10% FBS. Cells were grown at 37°C in a humidified atmosphere of 5% CO₂.

Renal tumor xenograft nude mice. QTRRE-2 and QTRRE-3 cell tumorigenicity in athymic National Cancer Institute female nude mice was performed as described previously (Patel *et al.*, 2003). Briefly, 5- to 6-week-old mice were purchased from Taconic (Hudson, NY) and were maintained on a 12-h light/dark cycle and allowed food and water *ad libitum*. The animals were divided into three groups ($n = 2$ per group). Once QTRRE-2 or -3 cells reached log phase growth stage in culture, they were harvested and resuspended in matrigel (BD Biosciences, San Jose, CA). The animals were injected subcutaneously at one site/animal with 5×10^6 cells/0.2 ml matrigel per site. When the size of a tumor reached 10–15 mm in diameter, the mice were euthanized, and sections of tumor were fixed in 10% phosphate-buffered formalin and embedded in paraffin or snap frozen in liquid nitrogen and stored at -80°C .

Animal dosing and tissue preparation. Male Eker rats (wild-type, *Tsc-2*^{+/+}, and mutant, *Tsc-2*^{EK/+}), 8 weeks old, were obtained from the University of Texas MD Anderson Cancer Center, Smithville, TX. The animals were housed according to a 12-h light/dark cycle and allowed food and water *ad libitum*. TGHQ was synthesized as previously described and used at > 98% purity, as determined by high performance liquid chromatography (Lau *et al.*, 1988). The rats were divided into four subgroups: (1) *Tsc-2*^{EK/+} control, (2) *Tsc-2*^{EK/+} TGHQ-treated, (3) *Tsc-2*^{+/+} control, and (4) *Tsc-2*^{+/+} TGHQ treated. The rats were administered TGHQ (2.5 μmol/kg in 0.5 ml of 1× PBS, ip) 5 days a week for 4 months, the dose increasing to 3.5 μmol/kg at 4 months for an additional 4 months, according to previously established protocol (Lau *et al.*, 2001). Control rats were administered PBS only. The TGHQ dosing solution was prepared fresh in 1× PBS daily. The animals were euthanized by CO₂ asphyxiation. For histological studies, a mid-sagittal longitudinal section of the left kidneys was fixed in 10% phosphate-buffered formalin and paraffin embedded. For biochemical assays, the outer stripe of the outer medulla (OSOM, regions from which tumors were derived), cortex, and renal tumors were excised, frozen immediately in liquid nitrogen, and stored at -80°C .

Nuclear and cytoplasmic extraction. Fractionation of (1) renal tumor xenografts derived from subcutaneous injection of QTRRE-2 or -3 cells in nude mice, (2) kidney sections from vehicle-treated nude mice, (3) renal tumors from TGHQ-treated *Tsc-2*^{EK/+} rats, (4) OSOM dissected from vehicle-treated *Tsc-2*^{EK/+} rats, or (5) HK2 cells was performed using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce Biotechnology, Rockford, IL) according to the manufacturer's protocol. For the fractionation of QTRRE-3 or HK2 cells, cells were seeded at a density of 3×10^5 cells per well in six-well plates. At 80–90% confluency, cells were treated with 3.6mM pentoxifylline or 3.3mM theophylline for 24 h in DMEM/F12 with 10% FBS or 50μM sorafenib for 1.5 h in DMEM/F12 with 2% FBS. Following drug treatments, cells were harvested with trypsin and washed with PBS. Cell fractionation was performed according to the manufacturer's protocol.

Western blot analysis. The OSOM of TGHQ or vehicle-treated *Tsc-2*^{EK/+} and *Tsc-2*^{+/+} rats, TGHQ-*Tsc-2*^{EK/+} rat renal tumors or QTRRE mouse xenograft tumors, and QTRRE-3 or HK2 cells were homogenized with 100 μl ice-cold lysis buffer (Cell Signaling Technology, Inc., Beverly, MA) containing 1mM Pefabloc SC (Roche) and Complete Protease Inhibitor Cocktail tablets (Roche). Protein lysates were then subjected to heat in a boiling water bath for 5 min prior to loading into 10 or 12% gels and were then separated via SDS-polyacrylamide gel electrophoresis (PAGE) with 10% resolving gels and 4% acrylamide stacking gels. Proteins were then transferred to polyvinylidene difluoride (PVDF; Bio-Rad Laboratories, Hercules, CA) membranes. Membranes were blocked with 5% milk. Primary antibodies used were cyclin D1 (A-12), B-Raf (H-145), Raf-1 (C-20), SP1 (E3), p27 (F-8), Rap1B (Santa Cruz Biotechnologies); p42/44, phospho-p42/44 (T202/Y204) (20G11) (Cell Signaling Technologies); and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Ambion, Austin, TX). The secondary immunoglobulin conjugated with horseradish peroxidase (Santa Cruz Biotechnology, CA) was used at a 1:3000 dilution. The blots were visualized with Amersham ECLTM Western Blotting

Detection Reagents (GE Healthcare, UK) and protein expression was determined by densitometric analysis with protein expression normalized to GAPDH.

Rap1 activation assay. Rap1 activation was determined as described (van Triest and Bos, 2004). The GST-Ral GDS-RBD fusion protein was isolated from *Escherichia coli* strain BL21. A 10% suspension of glutathione-agarose beads was pre-coupled to 100 μ l of cleared GST-Ral GDS-RBD lysate for 1 h on a tumbler at 4°C. HK2 cells and QTRRE-3 cells were treated with 3.6mM pentoxifylline (Sigma) for 24 h. Total cell lysates were isolated using Cell Lysis Buffer (Cell Signaling Technology, Inc., Beverly, MA). For each sample, equal quantities of total cell lysate were incubated with the GST-Ral GDS-RBD protein and glutathione-agarose beads slurry for 1.5 h on a tumbler at 4°C. After coupling, beads were washed 4 times with Cell Lysis Buffer and bound proteins were eluted in 15 μ l of XT Sample Buffer (Bio-Rad). Precipitates were subjected to 12% SDS-PAGE followed by transfer onto PVDF membranes, which were subsequently incubated overnight with a 1:1000 dilution of Rap1B (Santa Cruz Biotechnology), then washed and incubated with 1:3000 dilution of goat immunoglobulin conjugated with horseradish peroxidase (Santa Cruz Biotechnology, CA). The blots were visualized with Amersham ECLTM Western Blotting Detection Reagents (GE Healthcare, UK).

B-Raf and Raf-1 kinase activity assay. At 80–90% confluency, QTRRE cells were treated with 3.6mM pentoxifylline or 3.3mM theophylline for 24 h in DMEM/F12 with 10% FBS. Cells were lysed with Cell Lysis Buffer, as described above, and 500 μ g of total cell lysate was immunoprecipitated using B-Raf and Raf-1 polyclonal antibodies (Santa Cruz Biotechnology, CA) bound to protein A/G-agarose beads (Pierce Biotechnology Inc., IL). Kinase activity of the immunoprecipitates was determined using B-Raf or Raf-1 Kinase Cascade Assay Kits (Upstate Biotechnology) as previously reported (Yoon *et al.*, 2004) and according to the manufacturer's protocol. The incorporated [γ -³³P]ATP was quantified by liquid scintillation spectroscopy.

Immunostaining. QTRRE cells grown on glass coverslips were treated with 3.6mM pentoxifylline or 3.3mM theophylline for 24 h in DMEM/F12 with 10% FBS. Cells were fixed with 4% paraformaldehyde, permeabilized with acetone, and blocked with 10% goat serum in 1% BSA/PBS. Cells were incubated with anti-p27 (Santa Cruz Biotechnology, CA) overnight at 4°C, followed by incubation with secondary Alexa 546 goat anti-mouse IgG (Molecular Probes, Eugene, OR) and TO-PRO-3 (Invitrogen) for 1 h at room temperature (RT). Cells were mounted with vectashield (Vector Laboratories, Burlingame, CA). The slides were imaged using a 60 \times water immersion plan-apochromat objective on an LSM 510 multiphoton/confocal laser-scanning microscope (Carl Zeiss, Inc., Thornwood, NY).

siRNA transfection. QTRRE cells were transfected at 50–60% confluence. For QTRRE transfection, 100nM B-Raf, Raf-1, p27, or cyclin D1 ON_TAR-GETplus SMARTpool siRNA, or siCONTROL Non-Targeting siRNA #5 (Dharmacon RNA Technologies, NY) was combined with serum-free DMEM/F12 media (100 μ l) and incubated for 5 min at RT. In parallel, DharmaFECT #2 (5 μ l) was incubated in serum-free DMEM/F12 (200 μ l) for 5 min at RT. Small interfering RNA (siRNA) solution (100 μ l) was then combined with the DharmaFECT #2 solution (200 μ l) and incubated for 20 min at RT. The siRNA-DharmaFECT complex solution was added directly to each well, mixed gently, and incubated for 24, 48, 72, or 96 h at 37°C in a CO₂ incubator.

Statistics. Data are expressed as means \pm SD. Mean values were compared using a Student's *t*-test, two tails, with unequal variances.

RESULTS

TGHQ Increases p27 Levels in *Tsc-2^{+/+}* and *Tsc-2^{EK/+}* Rats and in QTRRE Tumor Xenografts from Nude Mice

Tsc-2^{EK/+} rats carry a single autosomal mutation on one allele of the tuberous sclerosis tumor suppressor gene that predisposes these rats to the development of spontaneous renal cell tumors,

and at a very high incidence (Eker *et al.*, 1981; Kobayashi *et al.*, 1995; Yeung *et al.*, 1994). The majority of renal cell tumors in *Tsc-2^{EK/+}* rats originate from within renal proximal tubules, and these tumors are histologically similar to renal tumors in humans (Everitt *et al.*, 1992; Walker, 1998). Western blot analysis was performed on the kidney lysates obtained from the OSOM region of the *Tsc-2^{EK/+}* and *Tsc-2^{+/+}* rats treated for 8 months with TGHQ or vehicle. Densitometric analysis of p27 expression, normalized to GAPDH, revealed that neither group exhibit detectable levels of p27 (Fig. 1A lanes 1–4). Interestingly, TGHQ treatment alone was sufficient to induce a substantial increase in p27 in the OSOM of 8-month *Tsc-2^{+/+}* rats (Fig. 1A lanes 5–6). An increase in p27 protein levels was observed within the OSOM of TGHQ-*Tsc-2^{EK/+}* rats treated with TGHQ for 8 months (Fig. 1A lane 7–8), and a substantial increase was seen in one of the renal tumors (Fig. 1A lane 9). Similarly, kidneys of vehicle-treated nude mice did not display detectable levels of p27 (Fig. 1B lane 2), but kidneys of nude mice with subcutaneous renal tumor xenografts showed a slight increase in p27 (Fig. 1B lane 1). Renal tumor xenografts derived from QTRRE cell lines express elevated levels of p27 (Fig. 1B lanes 3–6).

Loss of *Tsc2* Correlates with Cytoplasmic Localization of p27 and Cyclin D1 in *Tsc2* Null Cells and Renal Tumors

QTRRE cells, which are null for tuberlin, express high levels of pERK1/2 (Yoon *et al.*, 2004) and high levels of cyclin D1 and p27 relative to *Tsc-2^{+/+}* normal human kidney cells (HK2) or

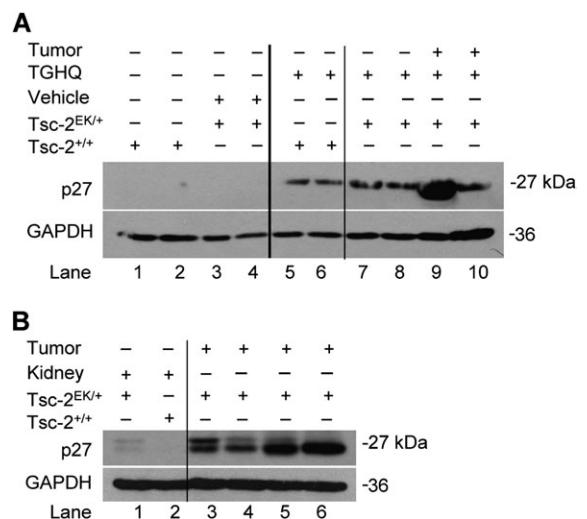


FIG. 1. Increased p27 levels in TGHQ-treated *Tsc-2^{+/+}* and *Tsc-2^{EK/+}* rats and QTRRE tumor xenografts in nude mice. (A) Whole tissue lysates from the OSOM or renal tumors of vehicle- or TGHQ-treated *Tsc-2^{+/+}* and *Tsc-2^{EK/+}* rats were immunoblotted for p27 and GAPDH. (B) Whole tissue lysates from the kidneys of nude mice treated with vehicle (lane 2) or with subcutaneous renal tumor xenografts (lane 1) and whole tissue lysates generated from renal tumor xenografts derived by subcutaneous injection of QTRRE cells in nude mice (lanes 3–6) were immunoblotted for p27 and GAPDH. Every other lane represents a duplicate animal from the same treatment.

Tsc-2^{+/+} porcine LLC-PK1 proximal tubule cells (Fig. 2A). We have previously shown that LLC-PK1 cells are an appropriate control model when compared with the QTRRE cells, with respect to the relevant signaling pathways following *Tsc-2* mutation (Yoon *et al.*, 2004). We analyzed the localization pattern of p27 in HK2 and QTRRE cells. Nuclear-cytoplasmic extraction of HK2 cells revealed nuclear localization of p27 and nondetectable levels of cyclin D1 (Fig. 2B), whereas in tumorigenic QTRRE cells, p27 and cyclin D1 were primarily localized to the cytosolic fraction (Fig. 2C). We next examined the cytosolic localization of p27 and cyclin D1 in renal tumors from TGHQ-treated *Tsc-2^{EK/+}* rats and in renal tumor xenografts formed following subcutaneous injection of QTRRE cells in nude mice. Nuclear-cytoplasmic extraction of the OSOM from vehicle-treated *Tsc-2^{EK/+}* rats did not display detectable levels of p27 or cyclin D1 (Fig. 2D) in either fraction. In contrast, renal tumors derived from *Tsc-2^{EK/+}* rats treated with TGHQ for 8 months revealed an increase in nuclear and cytoplasmic p27 (Fig. 2D). The cytoplasmic localization of p27 correlated with increased levels of cytosolic cyclin D1 (Fig. 2D). Similarly, renal tumor xenografts derived from QTRRE cells in nude mice expressed elevated levels of cytosolic and nuclear p27 (Fig. 2E) and an increase in cytoplasmic cyclin D1 (Fig. 2E). Kidneys from vehicle-treated nude mice did not display detectable levels of p27 or cyclin D1 (Fig. 2E lane 2) in either fraction.

cAMP-Mediated Rap1 and B-Raf Activation

Increases in cAMP have been implicated in the activation of Rap1 and in the subsequent downstream activation of B-Raf in

rat neuronal cells and in human acute megakaryoblastic leukemia cells (Garcia *et al.*, 2001; Grewal *et al.*, 2000; Vossler *et al.*, 1997). The influence of cAMP on Rap1 activation in QTRRE and HK2 cells was therefore examined, and consistent with other cell models, Rap1B-GTP was elevated in both QTRRE and HK2 cells following treatments that increase cAMP concentrations (Figs. 3A and 3B).

Rap1 can bind both B-Raf and Raf-1, but Rap1 binding results in activation of B-Raf and inhibition of Raf-1 (Dugan *et al.*, 1999; Garcia *et al.*, 2001; Okada *et al.*, 1998; Peyssonnaud and Eychene, 2001; Vossler *et al.*, 1997). B-Raf (Fig. 3C) and Raf-1 (Fig. 3D) activation by cAMP in QTRRE cells was determined by kinase-specific activity assays following phosphodiesterase inhibition with pentoxifylline or theophylline. Pentoxifylline increased B-Raf kinase activity by approximately 40% (Fig. 3C) but did not result in a statistically significant change in Raf-1 kinase activity (Fig. 3D). Similarly, cAMP preservation with theophylline resulted in a 50% increase in B-Raf kinase activity (Fig. 3C), and a nominal 9% increase in Raf-1 kinase activity (Fig. 3D).

B-Raf Regulates p27 Protein Levels

To investigate whether Raf kinases contribute to high p27 protein levels, p27 was determined by Western blotting following treatment of QTRRE cells with the Raf kinase inhibitor sorafenib (50 μ M). Sorafenib caused a time-dependent decrease in p27, with a 40% reduction by 90 min. There is a sustained decrease in pERK1/2 between 30 and 60 min with recovery beginning to occur around 90 min (Fig. 4A). The recovery of ERK phosphorylation is

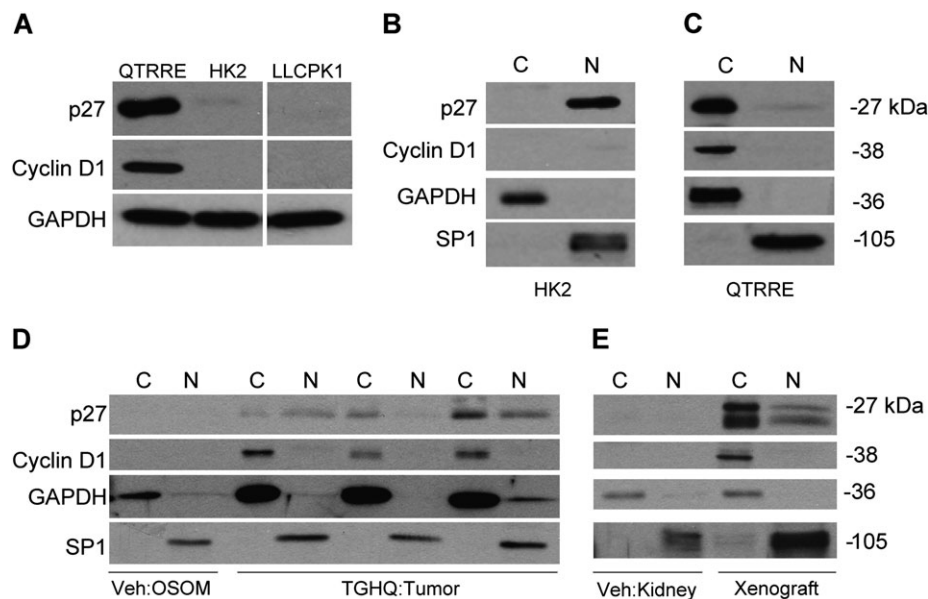


FIG. 2. Cytoplasmic localization of p27 in tuberin-null renal tumors. (A) Whole cell lysates from QTRRE, HK2, and LLC-PK1 cells were immunoblotted for p27, cyclin D1, and GAPDH. Cytosolic [C] and nuclear [N] lysates were generated from (B) HK2 cells, (C) QTRRE cells, (D) kidney OSOM of 8-month vehicle-treated *Tsc-2^{EK/+}* rats or tumors from 8-month TGHQ-treated *Tsc-2^{EK/+}* rats, and (E) vehicle-treated kidneys of nude mice or renal tumor xenografts derived by subcutaneous injection of QTRRE cells in nude mice. Samples were immunoblotted for p27, cyclin D1, GAPDH (cytosolic fractionation control), and SP1 (nuclear fractionation control).

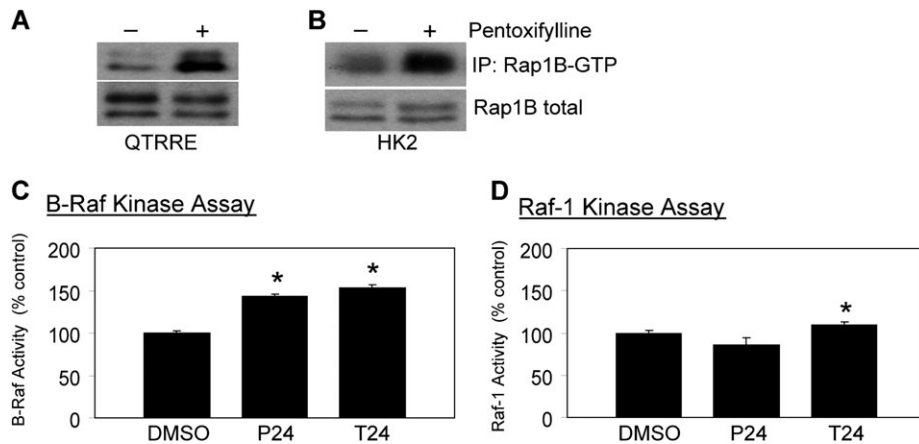


FIG. 3. Phosphodiesterase inhibition activates Rap1 and B-Raf in QTRRE cells. (A) QTRRE or (B) HK2 cells were treated with pentoxifylline (3.6mM) for 24 h. Rap1B-GTP was precipitated with GST-Ral GDS-RBD bound to glutathione-sepharose beads from 500 μ g of QTRRE or HK2 total cell lysate, and after removal of the beads, identified with a Rap1B antibody following SDS-PAGE and Western blotting. Rap1B immunoblots of the same total cell lysates were performed to confirm equal expression of total Rap1B between DMSO- and pentoxifylline-treated lysates. (C) and (D), QTRRE cells were treated with pentoxifylline (P, 3.6mM), theophylline (T, 3.3mM), or DMSO for 24 h. Equal amounts of lysates were subjected to (C) B-Raf kinase activity assay or (D) Raf-1 kinase activity assay. B-Raf and Raf-1 activity were determined as described in "Materials and Methods". Values represent the mean \pm SD ($n = 3$). A significant difference was seen between DMSO and pentoxifylline or theophylline treatments in QTRRE cells at $*p < 0.05$.

likely due to several factors, including changes in the protein stability via binding of p27 to cyclin D1 influencing the kinetics of ERK phosphorylation. Thus, although ERK phosphorylation appears to be recovering, the propagation of this signaling event may still be manifest.

Similar to most FDA approved small molecule inhibitors, sorafenib is not selective for its primary target, Raf kinases; in a kinase-binding assay, sorafenib bound 10% of 384 kinases tested, with affinities within tenfold of that for Raf kinases (Karaman *et al.*, 2008). Therefore, to confirm, as well as identify which Raf isoform is responsible for MAPK regulation of p27 protein levels, QTRRE cells were transfected with B-Raf and

Raf-1 siRNA. Real-time PCR analysis of both Raf isoforms, following 48 h siRNA treatment, revealed an approximate 95% decrease in Raf messenger RNA (mRNA) levels (data not shown). Western blot analysis revealed a 75% knockdown B-Raf concomitant with a 40% decrease in p27 protein levels (Fig. 4B). In contrast, almost complete knockdown of Raf-1 had no effect on p27 levels (Fig. 4C). Both Raf siRNA's were target specific, and neither Raf siRNA produced a significant change in GAPDH protein expression. These data, combined with the increase in Rap/B-Raf activation following cAMP stimulation in QTRRE cells (Fig. 3), prompted us to subsequently determine the connection between cAMP signaling and p27 expression.

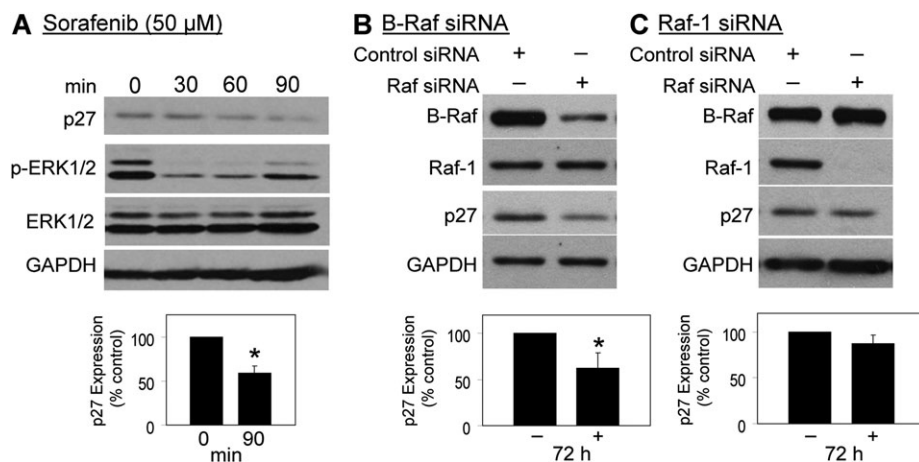


FIG. 4. Effect of sorafenib and Raf siRNA on p27 expression in QTRRE cells. (A) QTRRE cells were treated with sorafenib (50 μ M) for 30, 60, and 90 min. QTRRE cells were transfected with (B) 100nM B-Raf or (C) Raf-1 ON_TARGETplus SMARTpool siRNA or siCONTROL Non-Targeting siRNA #5 (Dharmacon RNA Technologies, NY) for 72 h. Whole cell lysates were subjected to Western blotting with antibodies specific to p27, pERK1/2, ERK1/2, and GAPDH. Values represent the mean \pm SD ($n = 3$). A significant difference in p27 protein levels was seen between control (DMSO) and sorafenib or B-Raf siRNA-treated cells at $*p < 0.01$.

cAMP Signaling Regulates Expression and Cytoplasmic Localization of p27

cAMP regulates p27 protein levels in a variety of cell types (Alderson and Hama, 2009; Bond *et al.*, 2008; da Silva *et al.*, 2008; Kowalczyk *et al.*, 2009; Paris *et al.*, 2006). To examine whether cAMP modulation influences p27 protein levels in QTRRE cells, we probed for p27 and p-ERK1/2 following treatment with phosphodiesterase inhibitors. Protein levels of p27 increased 2.8- and 2.2-fold following incubation with pentoxifylline (Fig. 5A) or theophylline (Fig. 5B), respectively, with an accompanying increase in the higher molecular weight band of p-ERK1/2. Pentoxifylline mediated increases in p27 were confirmed by confocal microscopy (Fig. 6). To investigate whether cAMP treatment can directly increase p27 protein levels, QTRRE cells were treated with dibutyryl cAMP for 19 h, which produced a 2.9-fold increase in p27 protein levels and a slight increase in the upper molecular weight band of p-ERK (Fig. 5C). Incubation with dibutyryl cAMP also caused a 1.4-fold increase in

cyclin D1 (Fig. 5C). Finally, sorafenib attenuated increased p27 and cyclin D1 levels after theophylline pretreatment by 57 and 48%, respectively (Fig. 5D).

To evaluate the effect of cAMP on the subcellular compartmental localization of p27, nuclear-cytoplasmic extraction of wild-type HK2 cells and tumorigenic QTRRE cells was performed. Treatment of HK2 cells with theophylline resulted in an increase in nuclear and cytosolic p27 and a modest increase in nuclear cyclin D1 (Fig. 7A). In contrast to HK2 cells, in QTRRE cells, pentoxifylline or theophylline caused an increase in cytosolic p27 (Fig. 7B). Furthermore, phosphodiesterase inhibition produced a corresponding increase in cytosolic cyclin D1 (Fig. 7B). Sorafenib treatment resulted in undetectable levels of nuclear p27 and a concomitant 25% decrease in cytosolic p27 (Fig. 7C) that corresponded with a 50% decrease in cytosolic cyclin D1 (Fig. 7C) as determined by densitometric analysis with protein expression normalized to GAPDH.

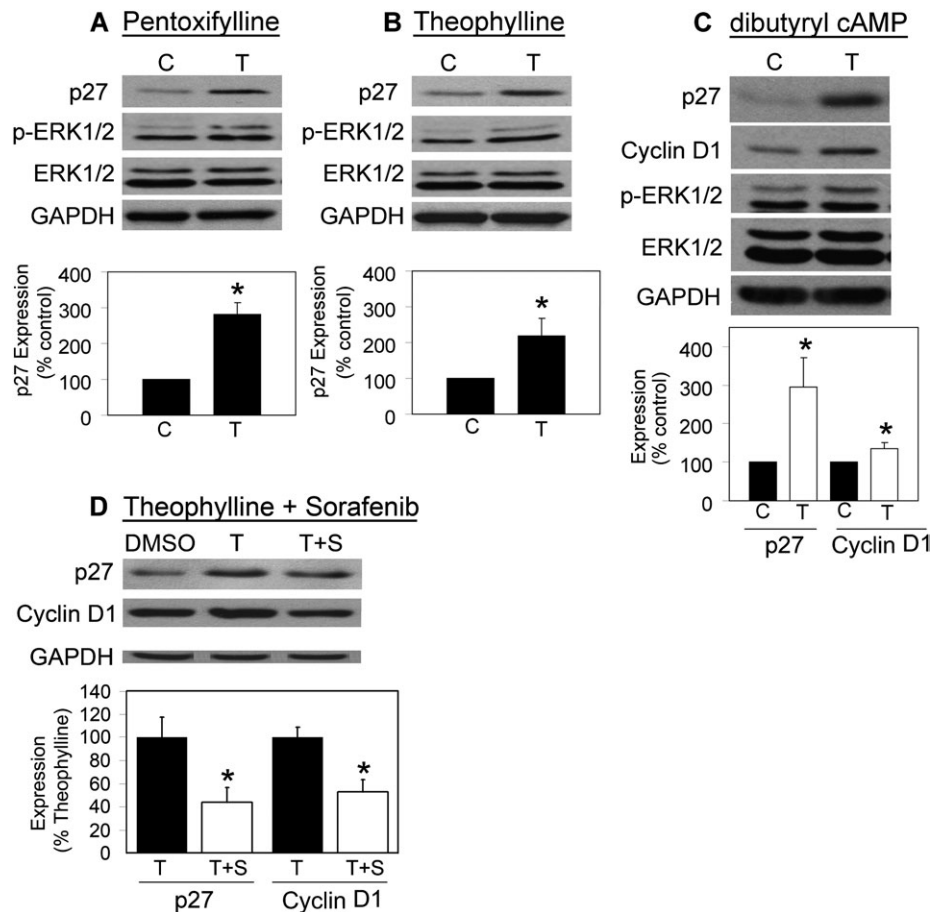


FIG. 5. Phosphodiesterase inhibition and dibutyryl cAMP regulates p27 expression and protein stability in QTRRE cells. QTRRE cells were treated with (A) pentoxifylline (3.6mM), (B) theophylline (3.3mM), (C) dibutyryl cAMP (1 mM), or (D) theophylline (3.3mM) for 24 h followed by sorafenib (50 μ M) for 90 mins. Cells treated with vehicle (DMSO or medium) served as controls. Equivalent whole cell lysates were subjected to Western blotting with antibodies specific to p27, cyclin D1, pERK1/2, ERK1/2, or GAPDH. Values represent the mean \pm SD ($n = 3$) for pentoxifylline and theophylline treatments and the mean \pm SD ($n = 4$) for dibutyryl cAMP. A significant difference in p27 and/or cyclin D1 protein levels was seen between DMSO (C) and pentoxifylline, theophylline, or dibutyryl cAMP treatments (T) in QTRRE cells at $*p < 0.05$.

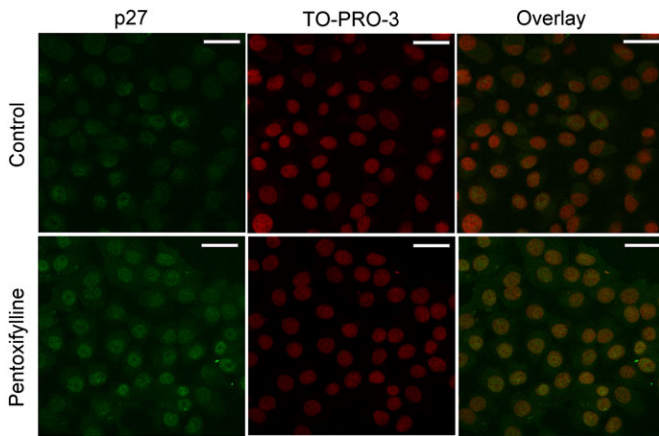


FIG. 6. Effect of pentoxifylline on p27 protein levels in QTRRE cells. QTRRE cells treated with pentoxifylline for 24 h were examined by immunocytochemistry with an anti-p27 antibody, TO-PRO-3 to detect nuclei, and an overlay of the two stains. Immunofluorescence was detected by confocal microscopy with a 60 \times water immersion plan-apochromat objective; white scale bars are 20 microns.

Cytoplasmic Stabilization of Cyclin D1 Is Codependent on p27 Cytosolic Relocalization

In MEFs, lack of p27 expression is associated with decreased cyclin D1 protein levels (Cheng *et al.*, 1999). Furthermore, p27 deficiency results in cyclin D1-CDK4 complex instability (Cheng *et al.*, 1999; Sherr and Roberts, 1999). To investigate which protein is a necessary component of p27-cyclin D1 cytosolic relocalization and stabilization, we knocked down p27 or cyclin D1 with siRNA. Following a 48-h treatment with p27 siRNA in QTRRE cells, there was an equivalent 88% decrease in both p27 and cyclin D1 protein levels (Fig. 8A); similarly, a 48-h treatment of cyclin D1 siRNA resulted in a 50% decrease in p27 protein levels (Fig. 8B).

DISCUSSION

We have established that modulation of B-Raf kinase activity directly alters p27 protein levels and promotes the cytoplasmic relocalization of p27 in TGHQ-mediated tuberous sclerosis

RCC. The cytoplasmic mislocalization and increased expression of both p27 and cyclin D1 were documented in (1) tuberin null renal tumors from TGHQ-treated *Tsc-2*^{EK/+} rats, (2) renal tumor xenografts derived by the subcutaneous injection of QTRRE (null of tuberin) cells in nude mice (Figs. 1 and 2), and (3) in tumorigenic QTRRE cells (compared to HK2 and LLC-PK1 cells) (Fig. 2). Moreover, treatment of *Tsc-2*^{+/+} rats for 8 months with TGHQ was sufficient to induce increased p27 expression within the OSOM (Fig. 1). Modulation of cAMP and MAPK cascades revealed that cAMP activation of Rap-GTP/B-Raf (Fig. 3) mediates increased p27 protein expression (Figs. 4–6) and its mislocalization to the cytoplasm (Fig. 7) in tuberin null QTRRE and human HK2 cells (Fig. 7). Finally, we established that p27-cyclin D1 complex formation stabilizes the proteins within the cytosol of QTRRE cells; siRNA targeting either p27 or cyclin D1 in QTRRE cells resulted in a substantial decrease in both proteins (Fig. 8). Similarly, manipulation of p27 protein levels in QTRRE cells by phosphodiesterase inhibitors (Figs. 5A and 5B and 7B) and dibutyryl cAMP (Fig. 5C) caused parallel increases in p27 and cyclin D1. Sorafenib caused parallel decreases in p27 and cyclin D1 in the absence (Figs. 4A and 7C) or presence of theophylline-enhanced cAMP effects (Fig. 5D).

Tuberin null renal tumors derived from Eker rats (*Tsc-2*^{EK/+}) (Yoon *et al.*, 2002), tuberin null QTRRE cells [44] and renal tumor xenografts derived from tuberin null QTRRE cells, all display cytoplasmic mislocalization of p27, consistent with reports that *Tsc2* null uterine leiomyomas and *Tsc2*^{-/-} MEFs display cytoplasmic localization of p27 (Short *et al.*, 2008). Tuberin has a dual function in the regulation of p27, regulating both its nuclear localization and stability. Tuberin complex formation with p27 inhibits 14-3-3-mediated cytoplasmic retention of p27 and promotes nuclear localization of p27 (Rosner *et al.*, 2007). Complex formation also stabilizes p27 protein levels by inhibiting degradation of p27 by the SCF-type E3 ubiquitin proteasome (Rosner and Hengstschlager, 2004). Consequently, loss of tuberin expression should increase degradation of p27. However, in contrast, the loss of tuberin in QTRRE cells and in renal tumors derived from TGHQ-treated *Tsc-2*^{EK/+} rats produced an unanticipated increase in

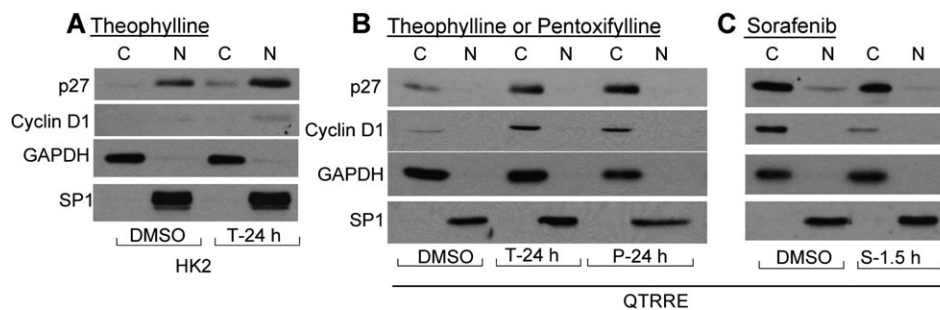


FIG. 7. Cytoplasmic mislocalization of p27 and cyclin D1 modulated by cAMP-MAPK signaling. (A) cytosolic [C] and nuclear [N] lysates were generated from HK2 cells treated with theophylline (3.3mM) or DMSO for 24 h. (B) and (C) cytosolic [C] and nuclear [N] lysates were generated from QTRRE cells treated with (B) theophylline (3.3mM), pentoxifylline (3.6mM), or DMSO for 24 h or (C) sorafenib (50 μ M) or DMSO for 1.5 h. Equal amounts of extracted lysates were subjected to SDS-PAGE and immunoblotting with antibodies specific to p27, cyclin D1, GAPDH (cytosolic fractionation control), or SP1 (nuclear fractionation control).

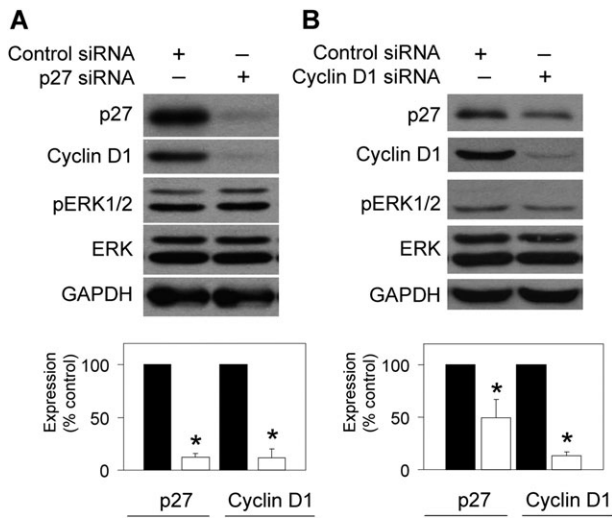


FIG. 8. p27 stabilizes cyclin D1 in the cytoplasm of QTRRE cells. QTRRE cells were transfected with (A) 100nM p27 ON_TARGETplus SMARTpool siRNA, (B) 100nM cyclin D1 ON_TARGETplus SMARTpool siRNA or siCONTROL Non-Targeting siRNA #5 (Dharmacon RNA Technologies, NY) for 48 h. Whole cell lysates were subjected to Western blotting with antibodies specific to p27, cyclin D1, pERK1/2, ERK1/2, and GAPDH. Values are means \pm SD ($n = 3$). A significant difference was seen between control siRNA and p27 siRNA-treated cells at $*p < 0.01$.

total p27 protein levels. We therefore suggest that p27-cyclin D1 complex formation in the cytoplasm protects p27 from targeted degradation by the SCF proteasome. The localization and degradation of p27 is complex and highly dependent on its phosphorylation status at a number of key residues (Vervoorts and Luscher, 2008). For example, in G_0 , phosphorylation on either S10 by dual specificity tyrosine phosphorylation-regulated kinase 1B (DYRK1B/MIRK) or T198 by AKT or ribosomal protein S6 kinase alpha-1/2 (RSK1/2) stabilizes p27 in the nucleus (Besson *et al.*, 2006; Deng *et al.*, 2004; Kossatz *et al.*, 2006; Kotake *et al.*, 2005). In G_1 , modification of p27 by AKT or RSK1/2 on T198 also allows for p27-cyclin D-CDK complex formation in the cytosol (Fujita *et al.*, 2002, 2003; Kossatz *et al.*, 2006). The modulation of these phosphorylation sites may be critical in cytoplasmic p27-cyclin D1 complex formation in RCC and must be further explored.

Treatment of tuberin null QTRRE cells with phosphodiesterase inhibitors or dibutyryl cAMP resulted in a parallel increase in p27 and cyclin D1 protein levels, suggesting that cAMP signaling is a key regulator of p27 expression and stabilization (Fig. 5). cAMP is an effective inducer of p27 expression in a variety of cell types, including schwannoma, vascular smooth muscle, neural, and skin cells (Alderson and Hama, 2009; Bond *et al.*, 2008; da Silva *et al.*, 2008; Kowalczyk *et al.*, 2009; Paris *et al.*, 2006). cAMP also regulates p27 transcription in neuronal cells (Shin *et al.*, 2009). Since phosphodiesterase inhibitors increased p27 levels in both tuberin negative and positive cells, tuberin status may be an independent factor in cAMP regulation of p27 expression.

Furthermore, in neuronal progenitor, retinal, schwannoma, and skin cells, a cAMP mediated increase in p27 promoted differentiation and/or cell cycle exit (Alderson and Hama, 2009; da Silva *et al.*, 2008; Kowalczyk *et al.*, 2009; Paris *et al.*, 2006). In contrast, increases in p27 in tuberin-deficient QTRRE cells did not result in cell cycle exit but rather an increase in the cytoplasmic p27/cyclin D1 complex. Therefore, concomitant increases in p27 and cyclin D1 suggest reciprocal protein stabilization. Moreover, p27 or cyclin D1 siRNA knockdown in QTRRE cells resulted in concomitant decreases in cyclin D1 or p27, respectively; which suggests increased protein stability via complex formation. When p27 is bound to cyclin D-CDK4 complexes, it is protected from degradation by the SCF^{SKP2} E3-ubiquitin-ligase complex (Kamura *et al.*, 2004). Similarly, due to the instability of the cyclin D1-CDK4 complex, p27 interaction is necessary for complex assembly and stabilization (Cheng *et al.*, 1999; Sherr and Roberts, 1999). Complexes of p27-cyclin D-CDK4 exhibit intact kinase activity (Blain *et al.*, 1997; James *et al.*, 2008) and may therefore play a crucial role in tumor formation.

We also show that phosphodiesterase inhibitors activate Rap-GTP and B-Raf kinase activity but have negligible effects on Raf-1 kinase activity (Fig. 3). These data are consistent with findings that Rap activation of ERK is dependent on B-Raf expression (Dugan *et al.*, 1999; Okada *et al.*, 1998; Schmitt and Stork, 2001). In B-Raf-negative NIH 3T3 cells, cAMP inhibition of the Raf-1/ERK MAPK cascade is mediated through Rap1 (Dugan *et al.*, 1999; Okada *et al.*, 1998; Schmitt and Stork, 2001). Therefore, we propose that cAMP is modulating the transcriptional activation of p27 through the Rap/B-Raf MAPK cascade (Fig. 9). Furthermore, an almost complete knockdown of Raf-1 with siRNA did not result in a significant change in p27 protein levels (Fig. 4C); these data, in combination with the Rap-GTP/B-Raf kinase activity assay (Fig. 3), strongly suggest that cAMP is only activating the B-Raf MAPK cascade and not Raf-1. In tumorigenic QTRRE cells, Raf-1 regulates cyclin D1 protein translation through ERK cross talk with eukaryotic translation initiation factor 4E-binding protein-1 (4EBP1) (Cohen, Gard, Nagle, Dietrich, Monks, and Lau, unpublished data). In contrast, inhibition of B-Raf has far less of an effect on cyclin D1 as Raf-1 and has no effect on 4EBP1 phosphorylation. Moreover, Raf inhibition with sorafenib did not cause a decrease in cyclin D1 mRNA levels, suggesting that protein translation and/or stability may be responsible for the observed decreases in cyclin D1 protein levels (Cohen, Gard, Nagle, Dietrich, Monks, and Lau, unpublished data). Consequently, we suspect that B-Raf regulates p27 transcription, and the increase in p27 protein also involves complex formation with cyclin D1 thereby inhibiting p27 degradation. These data highlight cAMP as an effective regulator of B-Raf MAPK-mediated induction of p27 protein levels in a tuberous sclerosis renal tumor model (Fig. 9).

The von Hippel-Lindau (*VHL*) tumor suppressor gene regulates cell cycle arrest in a manner similar to *Tsc-2*, via stabilization of p27^{Kip1} (Pause *et al.*, 1998). The *VHL* tumor

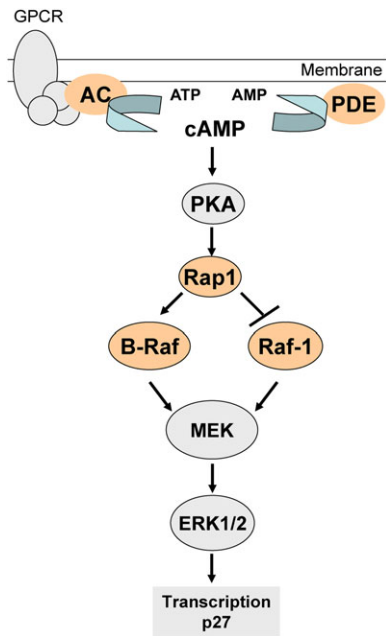


FIG. 9. Rap1-GTP/B-Raf MAPK regulation of p27 in QTRRE cells. Adenylate cyclase (AC) converts adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP), and phosphodiesterases (PDE) revert cAMP to AMP. cAMP mediates protein kinase A (PKA) activation of Rap1. Rap1-GTP activates B-Raf kinase activity and inhibits Raf-1. B-Raf phosphorylates and activates the MEK/ERK MAPK cascade. ERK1/2 regulates transcription of p27.

suppressor gene is a primary target for mutations in human RCC (Gnarra *et al.*, 1994; Shuin *et al.*, 1994) but does not contribute to rodent renal tumorigenesis (Kikuchi *et al.*, 1995; Walker *et al.*, 1996). *Tsc-2* is mutated in human (Wagner and Linehan, 1996; Yu *et al.*, 1986) and rodent (Kobayashi *et al.*, 1995, 1999; Onda *et al.*, 1999; Yeung *et al.*, 1994) RCC. Although *Tsc-2* is the primary target for RCC in our Eker rat model, both *Tsc-2* and *VHL* result in predominately clear cell RCC pathology and share converging signaling proteins, including p27. Recent developments have revealed the duality of p27 as both a tumor suppressor and as an oncogene in certain cancers (Sicinski *et al.*, 2007). As a tumor suppressor, reduced p27^{Kip1} levels in primary cancers are highly correlated with decreased patient survival (Short *et al.*, 2008; Sicinski *et al.*, 2007). This duality of p27 is also displayed in RCC. Over 370 human RCC tissues were immunostained with p27 to determine its subcellular localization; staining identified nuclear p27 in 78% of the tumors and cytoplasmic localization in 46% of the tumors (Pantuck *et al.*, 2007). Furthermore, cytoplasmic p27 staining was higher in metastatic RCC and was identified as a predictor of disease-specific survival time, with cytoplasmic mislocalization predicting a poor prognosis (Pantuck *et al.*, 2007). Therefore, the induction of p27 expression may have a variable outcome depending on the tumor grade.

Cytoplasmic mislocalization of p27 occurs in a variety of human cancers that correlate with a high tumor grade and poor survival rate (Alkarain *et al.*, 2004; Ciaparrone *et al.*, 1998; Hennenlotter *et al.*, 2008; Masciullo *et al.*, 2000; Motti *et al.*,

2005; Pantuck *et al.*, 2007; Singh *et al.*, 1998; Viglietto *et al.*, 2002). Depending on the tissue specificity and pathology of the tumor, p27 expression may be either low in the total cell lysate or elevated in the cytosol due to cytoplasmic localization (Short *et al.*, 2008; Sicinski *et al.*, 2007). Recently, transformed keratinocytes treated with phosphodiesterase inhibitors increased their sensitivity to growth inhibition by glucocorticoids (Kowalczyk *et al.*, 2009). The use of phosphodiesterase inhibitors as a method to induce p27 expression, and ultimately cell cycle exit, may be a useful tool to treat low-grade tumors but may exacerbate high-grade tumor formation. Therefore, further characterization of the relationship between cAMP pathway activation and p27 cytoplasmic localization will shed light on the role of p27 in renal tumor formation.

Our data identified B-Raf MAPK as an effective modulator of p27 expression, which furthers our understanding of the mechanisms of action of sorafenib in treating human RCC. Studies performed in human RCC 786-0 and Caki-1 cells suggest that neither *VHL* status nor the mammalian target of rapamycin (mTOR) cascade play a direct role in the cytoplasmic relocation of p27 (Kim *et al.*, 2009). Therefore, we suspect that the B-Raf/MAPK cascade is the primary pathway regulating p27 protein expression and cytoplasmic stabilization. Finally, the correlation between the cytoplasmic localization of p27 and an aggressive tumor type during human RCC highlights the necessity for an appropriate rodent model mirroring these characteristics. Our tuberin-deficient renal tumor models not only have similar clear cell pathology but also display cytoplasmic p27-cyclin D1, which makes it an ideal model to further characterize p27 as a predictive biomarker in RCC.

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