ERK Crosstalks with 4EBP1 to Activate Cyclin D1 Translation during Quinol-Thioether–Induced Tuberous Sclerosis Renal Cell Carcinoma

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The mammalian target of rapamycin (mTOR) and mitogen-activated protein kinase signaling cascades have been implicated in a number of human cancers. The tumor suppressor gene tuberous sclerosis-2 (Tsc-2) functions as a negative regulator of mTOR. Critical proteins in both pathways are activated following treatment of Eker rats (Tsc-2−/−) with the nephrocarcinogen 2,3,5-tris-(glutathion-S-yl)hydroquinone (TGHQ), which also results in loss of the wild-type allele of Tsc-2 in renal preneoplastic lesions and tumors. Western blot analysis of kidney tumors formed following treatment of Tsc−2−/− rats with TGHQ for 8 months revealed increases in B-Raf, Raf-1, pERK, cyclin D1, 4EBP1, and p-4EBP1-Ser65, -Thr70, and -Thr37/46 expression. Similar changes are observed following TGHQ-mediated transformation of primary renal epithelial cells derived from Tsc−2−/− rats (quinol-thioether rat renal epithelial [QTRRE] cells) that are also null for tuberin. These cells exhibit high ERK, B-Raf, and Raf-1 kinase activity and increased expression of all p-4EBP1s and cyclin D1. Treatment of the QTRRE cells with the Raf kinase inhibitor, sorafenib, or the MEK1/2 kinase inhibitor, PD 98059, produced a significant decrease in the protein expression of all p-4EBP1s and cyclin D1. Following siRNA knockdown of Raf-1, Western blot analysis revealed a significant decrease in Raf-1, cyclin D1, and all p-4EBP1 forms noted above. In contrast, siRNA knockdown of B-Raf resulted in a nominal change in these proteins. The data indicate that Raf-1/MEK/ERK participates in crosstalk with 4EBP1, which represents a novel pathway interaction leading to increased protein synthesis, cell growth, and kidney tumor formation.

Key Words: Raf-1; phosphorylation; MAPK; renal cell carcinoma; quinol-thioether.

Hyperphosphorylation of the eukaryotic translation initiation factor 4E (4E-binding protein 1, 4EBP1) is associated with a variety of high-grade tumors (Kremer et al., 2006; Rojo et al., 2007). Hypophosphorylated 4EBP1 binds to eukaryotic translation initiation factor 4E (eIF4E), blocking eIF4E-complex cap-dependent initiation of translation (Gingras et al., 1999). The hierarchical phosphorylation of 4EBP1, on four proline-directed sites (ser/thr-pro), suppresses 4EBP1’s ability to bind and inhibit eIF4E, allowing for the formation of the eIF4E complex and subsequent initiation of translation (Beugnet et al., 2003; Gingras et al., 1999, 2001; Huang and Manning, 2008). Phosphorylation of 4EBP1 is abrogated by inhibition of the mammalian target of rapamycin complex 1 (mTORC1), comprised of TOR, Raptor, and LST8, which is highly sensitive to inhibition by rapamycin (Beretta et al., 1996; Beugnet et al., 2003; Gingras et al., 1999, 2001; Huang and Manning, 2008; Shaw and Cantley, 2006). Inhibition of mTORC1 is intricately regulated by the tuberous sclerosis Tsc-1/Tsc-2 protein complex (Huang and Manning, 2008). Tuberous sclerosis is an autosomal dominant hereditary disease associated with the formation of hamartomas in multiple organs and extensive renal disease (Okada et al., 1982; Osborne et al., 1991; Stillwell et al., 1987). Germline mutations in the Tsc-1 and Tsc-2 genes result in alterations in cell growth, survival, proliferation, migration, differentiation and angiogenesis (Krymskaya, 2003; Yeung, 2003). Rats lacking one of the normal Tsc-2 alleles (Eker rats) are predisposed to the development of renal tumors (Eker and Mossige, 1961).

The gene products of Tsc-1 (hamartin) and Tsc-2 (tuberin) form a heterodimeric protein complex that negatively regulates the mTOR signaling pathways. Inhibition of mTOR by tuberin occurs through the GTPase-activating protein (GAP) activity of tuberin toward the small G protein Rheb, which acts upstream of mTOR (Inoki et al., 2003; Long et al., 2005; Tee et al., 2003). Loss of tuberin expression results in increased levels of Rheb-GTP and subsequently activation of mTOR. Rheb-GTP/mTOR signaling has been implicated in tumor development, via its downstream modulation of protein synthesis, cell proliferation, cell cycle progression, and cell survival (Huang and Manning, 2008; Jiang and Liu, 2008). In one model, activated Rheb-GTP was found to inhibit Raf-1 and B-Raf.
serine/threonine kinases in noncarcinogenic human embryonic kidney (HEK293) cells, following siRNA knockdown of tuberin (Karbowiak et al., 2004). In contrast, utilization of the Eker rat (Tsc-2<sup>EK+/+</sup>) has revealed a role for tuberin as a suppressor of mitogen-activated protein kinase (MAPK) signaling during renal carcinogenesis. The Eker rat (Tsc-2<sup>EK+/+</sup>) is a derivative of the Long-Evans strain, bearing a mutation in one allele of the Tsc-2 gene, which predisposes these animals to renal cancer (Eker and Mossige, 1961; Everitt et al., 1992; Kobayashi et al., 1995; Walker et al., 1992; Yeung et al., 1993). A germline insertion of an endogenous retrovirus in the Tsc-2 gene is responsible for the predisposing Eker mutation (Hino et al., 1995; Yeung et al., 1994). Treatment of wild-type (Tsc-2<sup>+/+</sup>) and mutant (Tsc-2<sup>EK+/+</sup>) Eker rats with 2,3,5-tris-(glutathion-S-yl)hydroquinone (TGHQ), a potent nephrotoxic (Peters et al., 1997) and nephrocarcinogenic (Lau et al., 2001) metabolite of hydroquinone, induces preneoplastic lesions, including toxic tubular dysplasias, and increases in the incidence of renal tumors only in animals carrying the mutant Tsc-2<sup>EK+/+</sup> allele (Lau et al., 2001). Loss of heterozygosity (LOH) at the Tsc-2 locus occurs in tumors and toxic tubular dysplasias, both of which exhibit elevated ERK kinase activity, consistent with TGHQ-induced loss of tumor suppressor function of the Tsc-2 gene (Lau et al., 2001; Yoon et al., 2002).

Additionally, TGHQ is mutagenic and can transform primary renal epithelial cells isolated from Eker rats in vitro, giving rise to the quinol-thioether rat renal epithelial (QTRRE) cell lines (Yoon et al., 2001). These cells are tuberin null due to LOH at the Tsc-2 gene locus (Yoon et al., 2001) and give rise to tumors when subcutaneously injected into athymic nude mice (Patel et al., 2003). QTRRE cells exhibit elevated ERK, B-Raf, and Raf-1 kinase activity (Yoon et al., 2002, 2004). Restoration of tuberin expression in QTRRE cells decreases ERK, B-Raf, and Raf-1 activity, suggesting that tuberin negatively modulates the Raf/MEK/ERK pathway upstream of Raf (Yoon et al., 2002, 2004). Recently, we reported that Rap1B preferentially modulates B-Raf, and not Raf-1, to regulate ERK transcription of p27 and cytoplasmic mislocalization of p27<sup>pkip</sup>-cyclin D1 in tuberin null QTRRE cells (Cohen et al., 2011). Therefore, in tuberous sclerosis renal cell carcinoma (RCC), Rap-GTP activation can regulate the B-Raf/MEK/ERK MAPK cascade, which provides further evidence that ERK regulation lies downstream of tuberin.

The Rafs regulate cellular proliferation and differentiation through the Raf/MEK/ERK/MAPK signaling cascade, which is hyperactivated in ~30% of all cancers (Cook et al., 1993; Hu et al., 1997). In an in vitro kinase assay, recombinant 4EBP1 was a substrate for ERK/MAPK, suggesting that ERK may play a role in the hierarchical phosphorylation of 4EBP1 (Haystead et al., 1994). The LOH in the Tsc-2 gene and subsequent loss of tuberin expression observed in TGHQ-induced renal tumors (Lau et al., 2001; Yoon et al., 2002), and tumorigenic QTRRE cells (Yoon et al., 2001), makes this a unique model to study the role of constitutive Raf/ERK MAPK in tuberin-deficient renal carcinogenesis. In the present study, we examine the relationship between tuberin, the B-Raf/ Raf-1/ERK MAPK cascade, and 4EBP1 hyperphosphorylation. Our findings identify, for the first time, Raf-1 as an effective regulator of 4EBP1 phosphorylation and activator of cap-dependent translation during renal carcinogenesis.

**MATERIALS AND METHODS**

**Animal dosing and tissue preparation.** Male Eker rats (wild-type, Tsc-2<sup>+/+:</sup>, and mutant, Tsc-2<sup>EK+/+</sup>) 8 weeks old, were obtained from the University of Texas MD Anderson Cancer Center, Smithville, TX. The animals were housed according to a 12:12 light-dark cycle and allowed food (standard laboratory rat chow) 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<sup>EK+/+</sup> control, (2) Tsc-2<sup>EK+/+</sup> TGHQ treated, (3) Tsc-2<sup>+/+</sup> control, and (4) Tsc-2<sup>+/+</sup> 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; then increased to 3.5 μmol/kg for 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), cortex, and renal tumors were excised, frozen immediately in liquid nitrogen, and stored at −80°C.

**Cell cultures.** The tuberin-negative cell line, QTRRE was established from primary renal epithelial cells (Yoon et al., 2001). HK2 (human kidney) cells were from American Type Culture Collection (Manassas, VA). HK2 and QTRRE cells were grown in Dulbecco’s modified Eagle medium: nutrient mixture F-12 (DMEM/F-12) (1:1) (Invitrogen) with 10% fetal bovine serum (FBS). Cells were grown at 37°C in a humidified atmosphere of 5% CO2.

**Immunohistochemistry.** The formalin-fixed paraffin-embedded kidney sections were sliced into 5 μm sections, deparaffinized in xylene, and rehydrated with decreasing concentrations of ethanol. The sections were incubated in 3% hydrogen peroxide to quench endogenous peroxidase activity. Next, the slides were placed in 10mM sodium citrate buffer (pH 6.0) and heated at 100°C to unmask antigens. The slides were then incubated with anti-cyclin D1 antibody (A-12) (Santa Cruz Biotechnology, Inc.) 1:200 dilution for 1 h, followed by incubation with biotin-conjugated secondary antibody, AB enzyme reagents, peroxide substrate, and 3,3′-diaminobenzidine solution provided in the ABC staining kit (Santa Cruz Biotechnology, Inc.). Counterstaining was performed with hematoxylin. Color was fixed with acid alcohol and dehydration steps. Slides were mounted with Permount mounting media.

**MTS proliferation assay.** QTRRE cells were seeded at 3.1 × 10<sup>4</sup> cells per well in 96-well flat-bottomed plates in DMEM/F12 with 10% FBS. At 80–90% confluency, cells were treated with 50μM sorafenib or PD 98059 in DMEM/F12 with 2%/FBS for 5, 25, 60, 90 min, and 24 h. Following sorafenib (synthesized at University of Arizona) or PD 98059 (Sigma) incubation, cells were washed twice with treatment media [DMEM (+) phenol red, (−) Na pyruvate, (+) 25mM Hepes, (+) l-glutamine (Invitrogen), and the proliferative activity of cells was determined by the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (CellTiter 96 AQueous Non-Radioactive Cell Proliferation Assay; Promega) according to the manufacturer’s recommendations. In metabolically active cells, MTS was bioreduced by dehydrogenase enzymes into a formazan product that was soluble in tissue culture medium. The absorbance of the formazan at 490 nm was measured in a SpectraMax M2 (Molecular Devices) 96-well plate reader. Values represent means ± SD (n = 6).

**Neutral red lysosomal membrane integrity assay.** QTRRE cells were seeded at a density of 5 × 10<sup>4</sup> cells per well in 24-well plates. At 80–90%
confluency, cells were treated with 50\(\mu\)M sorafenib or PD 98059 in DMEM/F12 with 2\% FBS for 0.5, 1, 1.5, 2, and 24 h. Following dosing, cells were washed with Hank’s balanced salt solution and then incubated with 50 \(\mu\)g/ml neutral red (NR) solution in DMEM (no phenol red) for 1 h at 37\(^\circ\)C/5\% CO\(_2\). The NR solution was removed and cells were washed with fixation solution (1% formaldehyde/1% CaCl\(_2\) mixture) for 2 min. This was followed by extraction using 1% glacial acetic acid/50\% ethanol solution for 15 min at room temperature (RT) in the dark. NR dye accumulation in lysosomes was assessed by measuring the absorbance at 540 nm. Values represent means ± SD (\(n = 4\)).

**Trypan blue cell viability assay.** QTRRE cells were seeded at a density of 2.5 × 10\(^5\) cells per well in 12-well plates. At 80–90\% confluency, cells were treated with 50\(\mu\)M sorafenib or PD 98059 in DMEM/F12 with 2\% FBS for 0.5, 1, 2, and 24 h. Following treatment, cells were washed with PBS, incubated with 10\% trypan blue for 5 min at 37\(^\circ\)C/5\% CO\(_2\), and trypan blue solution quenched with DMEM/F12 with 10\% FBS. Detached cells were mixed with equal volume of trypan blue solution (cell grow) and counted on a haemocytometer. Values represent means ± SD (\(n = 3\)).

**siRNA transfection.** QTRRE or HK2 cells were seeded at a density of 3 × 10\(^5\) cells per well in six-well plates. When cells were 50–60\% confluent, each well was replaced with 1.7 ml DMEM/F12 with 10\% FBS. For QTRRE transfection, 100\(\mu\)M B-Raf or Raf-1 ON_TARGETplus SMARTpool siRNA or siCONTROL non-targeting siRNA (Dharmacon RNA Technologies) was combined with 100 \(\mu\)l of serum-free DMEM/F12 media and incubated for 5 min at RT. For HK2 transfection, 50\(\mu\)M of Tsc2 ON_TARGETplus SMARTpool siRNA or siCONTROL non-Targeting siRNA pool (Dharmacon RNA Technologies) was combined with 100 \(\mu\)l of serum-free DMEM/F12 media and incubated for 5 min at RT. In parallel, 5\(\mu\)l of DharmaFECT #2 (QTRRE) or DharmaFECT #1 (HK2) was incubated in 200 \(\mu\)l serum-free DMEM/F12 for 5 min at RT. siRNA solution (100 \(\mu\)l) was then combined with the 200 \(\mu\)l DharmaFECT #2 (QTRRE) or #1 (HK2) solution 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\(^\circ\)C in a CO\(_2\) incubator.

**Real-time PCR determination of B-Raf and Raf-1.** Total RNA from B-Raf, Raf-1, or control siRNA transfected in QTRRE cells were isolated with TRI Reagent (Sigma) utilizing the manufacturer’s protocol, and 4.5 \(\mu\)g RNA, in a 20 \(\mu\)l total reaction volume, was reverse transcribed using the Fermentas First Strand cDNA Synthesis kit according to the manufacturer’s protocol. PCR products were generated using the Advantage cDNA PCR kit (Clontech) according to manufacturer’s protocol. Amplicon sizes and primers were as follows: 233 bp cyclin D1 (forward: 5'-GGC TAC CCT GAC ACC AAT CT-3'; reverse: 5'-GGC TCC AGA GAC AAG AAA CG-3') and 228 bp actin-beta (forward: 5'-AGC CAT GTA CGT AGC CAT CC-3'; reverse: 5'-CTC GCT GTG GTG AA-3'). The thermocycling conditions for cyclin D1 were 95\(^\circ\)C for 5 min, then 28 cycles of 95\(^\circ\)C for 30 s, 59\(^\circ\)C for 40 s, and 72\(^\circ\)C for 60 s, followed by a 10 min 72\(^\circ\)C final extension. The thermocycling conditions for GAPDH were 95\(^\circ\)C for 2 min; then 26 cycles of 95\(^\circ\)C for 15 s, 55\(^\circ\)C for 30 s, and 72\(^\circ\)C for 30 s, followed by a 10 min 72\(^\circ\)C final extension. PCR products were separated on 2\% ethidium bromide-stained agarose gel. Each PCR reaction was repeated twice to confirm reproducibility.

**Statistics.** Data are expressed as means ± SD. Mean values were compared using a Student’s \(t\)-test, two tails, with equal variances.

## RESULTS

### TGHQ-Induced Expression of Cyclin D1

Cyclin D1 facilitates tumor growth by promoting the transition from G\(_0\)/G\(_1\) to S phase and by modulating a number of proteins involved in transcriptional activation (Alao, 2007; Coqueret, 2002). Loss of tuberin expression results in an increase in cyclin D1 protein expression, inducing quiescent cells to enter S phase and, subsequently, an increase in proliferation (Soucek et al., 1997, 1998; Yoon et al., 2002). In the initial TGHQ bioassay study, we reported that physiological stress from intraperitoneal saline injections induced a modest upregulation of tuberin, a conditional negative regulator of cell proliferation, in the 4-month treated rats within the OSOM but no effect within the cortex (Yoon et al., 2002). In the present study, we investigated the expression of cyclin D1 protein in TGHQ-treated rats. Each treatment group has essentially equivalent levels of cyclin D1 expression, but there does appear to be a minor increase in cyclin D1 in 4-month vehicle-treated control rats (lane 3, Fig. 1A) when compared with the naïve Eker rats, which may be related to animal handling and the subsequent stress response in the initial periods of the study (4 months), which by 6- to 8-month subsides (as quantified by densitometry, data not shown). Western immunoblotting demonstrated that cyclin D1 protein expression maintained a modest increase in the OSOM until 6 months of TGHQ treatment (Fig. 1B), followed by substantial increases in renal tumors in the 8-month TGHQ-treated Tsc\(_{2}\)E2K\(/+\) rats (Fig. 1C). In both the 6- and 8-month treatment groups, cyclin D1 expression was elevated more in the OSOM of TGHQ-Tsc\(_{2}\)E2K\(/+\) rats than TGHQ-Tsc\(_{2}\)E2K\(/-\) rats. Immunohistochemical
IHC staining revealed that cyclin D1 is increased in proximal tubules of Tsc-2\(^{+/+}\) and Tsc-2\(^{Eki/+}\) rats treated with TGHQ for 4 months (Fig. 2). Compared with saline-treated animals, IHC staining for cyclin D1 was greater in Tsc-2\(^{Eki/+}\) rats relative to Tsc-2\(^{+/+}\) (Fig. 2). The differences in the observation of increases in cyclin D1 between the Western and IHC analyses is probably simply due to differences in the sensitivity of the two assays. These results are in keeping with previous findings that cell proliferation per se is necessary, but not sufficient for tumor development, and that additional genetic alterations (i.e., loss of the wild-type Tsc-2 allele) play an important role in TGHQ-induced nephrocarcinogenesis (Yoon et al., 2002).

**Increases in B-Raf/Raf-1/p-ERK Kinases during Renal Tumor Development**

The ser/thr kinase Raf-1 is ubiquitously expressed in all tissues and cell types, but B-Raf has restricted expression (Wellbrock et al., 2004). Each Raf isoform is differentially and dynamically activated, carrying out distinct functions in cells (Garnett et al., 2005). Western blot analysis of the OSOM of 4- or 6-month vehicle- or TGHQ-treated Tsc-2\(^{Eki/+}\) and Tsc-2\(^{+/+}\) rats revealed that B-Raf expression was negligible compared with 8 months of TGHQ treatment (Figs. 3A and B); but there was significant induction of B-Raf protein in a spontaneous tumor formed in a 6-month vehicle-treated Tsc-2\(^{Eki/+}\) rat (Fig. 3A). Although B-Raf expression was barely detectable in the OSOM, of the animals treated for 4 months, even following overexposure of the film, there does appear to be only a minor increase in B-Raf in vehicle-treated rats (lane 3 and 4, Fig. 3A) when compared with the naïve

![FIG. 1. TGHQ induces cyclin D1 expression in OSOM and renal tumors from Tsc-2\(^{Eki/+}\) rats. Cyclin D1 was visualized by Western blot analysis of tissue obtained from Tsc-2\(^{+/+}\) (wild-type) or Tsc-2\(^{Eki/+}\) (mutant) rats treated with TGHQ or saline for (A) 4 (B) 6, or (C) 8 months (OSOM and renal tumor). GAPDH (bottom panels) (A–C) was used as a loading control. n = 3 rat kidneys per group, representative OSOM or renal tumor sample displayed.](image1)

![FIG. 2. Histochemistry of cyclin D1 reactivity in renal tissue. Kidney slices immunoblotted for cyclin D1, from (A) Tsc-2\(^{+/+}\) rats treated with saline, (B) Tsc-2\(^{Eki/+}\) rats treated with saline, (C) Tsc-2\(^{+/+}\) treated with 2.5 \(\mu\)mol/kg (ip) of TGHQ for 4 months, and (D) Tsc-2\(^{Eki/+}\) rats treated with 2.5 \(\mu\)mol/kg (ip) of TGHQ for 4 months. Scale bars, 200 \(\mu\)m.](image2)
Eker rat (lanes 1 and 2, Fig. 3A), which may be related to animal handling and the subsequent stress response during the initial periods of the study (4 months), which by 6-month subsides (Fig. 3B ). Western blot analysis of the OSOM of 8-month vehicle control 
\( T_{sc-2}^{+/-} \) and 
\( T_{sc-2}^{E+/+} \) rats revealed that neither animal had detectable levels of B-Raf, but both expressed equivalent levels of Raf-1 (Fig. 3B). Although Raf-1 induction was observed within 8-month TGHQ-
\( T_{sc-2}^{E+/+} \) OSOM and renal tumors, there was a more substantial induction of B-Raf (Fig. 3B). Nonspecific binding of the B-Raf antibody in tissue prevented us from obtaining IHC data.

A previous study demonstrated that renal tumors derived from TGHQ-treated 
\( T_{sc-2}^{E+/+} \) rats had significantly elevated ERK activity compared with the OSOM tissue from all other treatment groups (Yoon et al., 2002). To confirm that the induction of Raf proteins in TGHQ tumors corresponds with an increase in MAPK activity, we probed for pERK by Western blot analysis. Concomitant with the increase in B-/Raf-1 protein expression, there was a parallel increase in pERK1/2 expression in TGHQ-
\( T_{sc-2}^{E+/+} \) tumors; with little observed change in total ERK1/2 or GAPDH (Fig. 3C).

Recent evidence indicates that high p-4EBP1 expression in renal tumors is associated with poor prognosis (Castellvi et al., 2006; Kremer et al., 2006; Rojo et al., 2007). To determine whether 4EBP1 hyperphosphorylation occurs during TGHQ-induced nephrocarcinogenicity, p-4EBP1 expression was determined by Western blot analysis in renal tumors derived from 8-month TGHQ-treated 
\( T_{sc-2}^{E+/+} \) rats and in the 8-month OSOM. Interestingly, the OSOM of all treatment groups did not express detectable levels of 4EBP1 and p-4EBP1-Ser65, -Thr70, and -Thr37/46 (Fig. 4). In contrast, renal tumors derived from TGHQ-treated 
\( T_{sc-2}^{E+/+} \) rats expressed high levels of total 4EBP1 with three distinct species at 15, 18, and 20 kDa. Moreover, high levels of various posttranslationally modified species of 4EBP1 and p-4EBP1 were observed (Fig. 4).

Sorafenib Treatment Decreases Mitochondrial Dehydrogenase Enzyme Activity and Lysosomal Membrane Integrity in QTRRE Cells

The small molecule inhibitor sorafenib is a new cancer therapeutic utilized in the treatment of advanced RCC. Sorafenib is a dual-action Raf kinase and vascular endothelial growth factor receptor inhibitor that targets tumor cell proliferation and tumor angiogenesis (Wilhelm et al., 2004). Mitochondrial dehydrogenase enzyme activity was quantified...
using the MTS assay, which measures the reduction of MTS to formazan. Lysosomal membrane integrity was measured by NR dye uptake and dye accumulation in lysosomes (Fig. 5A). Treatment of QTRRE cells with sorafenib resulted in a sustained decrease in both cellular mitochondrial dehydrogenase enzyme activity and lysosomal membrane integrity over the 2-h time course, with a maximum decrease of greater than 80% by 24 h (Fig. 5A). Although mitochondrial dehydrogenase enzyme activity and lysosomal membrane integrity were severely impaired, by 65 and 75%, respectively, following 1.5 or 2 h of sorafenib treatment, only a 20% decrease in cellular membrane integrity was measured by the trypan blue assay (Fig. 5A). By 24 h, a 90% loss of cell viability was observed (Fig. 5A). Thus, decreases in mitochondrial dehydrogenase enzyme activity and lysosomal membrane integrity precede overt cell death. Conversely, treatment of QTRRE cells with PD 98059, a MEK1/2 kinase inhibitor, resulted in a nominal but significant decrease in mitochondrial dehydrogenase enzyme activity after 5 and 25 min but complete metabolic recovery occurred by 90 min (Fig. 5B). Furthermore, there was no significant decrease in lysosomal membrane integrity (NR assay) or cellular membrane integrity (trypan blue assay) over the 24-h time course following PD 98059 treatment (Fig. 5B).

Raf-1 Regulates Hyperphosphorylation of 4EBP1 Protein Levels

In parallel with the p4EBP1 Western analysis of renal tumors derived from TGHQ-treated Tsc-2\(^{2Ek/+}\) rats, TGHQ-treated Tsc-2\(^{2Eko/+}\), -Thr70, and -Thr37/46 were visualized by Western blot analysis of OSOM or renal tumor tissue obtained from Tsc-2\(^{2Eko/+}\) or Tsc-2\(^{2Ek/+}\) rats treated with TGHQ or saline for 8 months. GAPDH was used as a loading control. n = 3 rat kidneys per group, representative OSOM or renal tumor sample displayed.

FIG. 4. 4EBP1 and p-4EBP1 are upregulated in renal tumors from TGHQ-treated Tsc-2\(^{2Ek/+}\) rats. 4EBP1 and p-4EBP1-Ser65, -Thr70, and -Thr37/46 were visualized by Western blot analysis of OSOM or renal tumor tissue obtained from Tsc-2\(^{2Eko/+}\) or Tsc-2\(^{2Ek/+}\) rats treated with TGHQ or saline for 8 months. GAPDH was used as a loading control, n = 3 rat kidneys per group, representative OSOM or renal tumor sample displayed.

FIG. 5. Effect of sorafenib or PD 98059 on metabolic activity, lysosomal membrane integrity, and membrane integrity in QTRRE cells. QTRRE cells were treated with 50\(\mu\)M (A) sorafenib or (B) PD 98059 for various time points as indicated. Control cells were treated with an equal volume of DMSO. Mitochondrial dehydrogenase activity (○) of QTRRE cells affected by sorafenib or PD 98059 was quantitated using the CellTiter 96 AQueous Non-Radioactive Cell Proliferation (MTS) Assay (Promega). Values represent the mean ± SD (n = 6). Lysosomal membrane integrity (●) was determined by measuring NR accumulation in QTRRE cells at 540 nm. Values represent the mean ± SD (n = 4). Plasma membrane integrity (□) in QTRRE cells was measured by trypan blue exclusion. Values are means ± SD (n = 3). A significant difference was seen between control (DMSO) and sorafenib- or PD 98059-treated cells at \(*p < 0.01\).
15 min after sorafenib treatment and was sustained for a further 45 min; by 90 min, a return to the pretreatment pattern of expression was observed (Fig. 6A). Interestingly, sorafenib treatment resulted in a sustained increase in a “faster migrating” band when probed for total 4EBP1 protein (Fig. 6A), and PD 98059 treatment resulted in the appearance of a third “faster migrating” species, although the appearance of this species was only transient in nature (Fig. 6B). The maximal effect of PD 98059 on the pattern of p-4EBP1 expression was observed at 25 min, with recovery occurring by 40 min (Fig. 6B). The effects of PD 98059 were transient compared with sorafenib because PD 98059 is a type 1 competitive inhibitor and sorafenib is a type 2 allosteric inhibitor. The on/off rate of competitive inhibitors is usually very fast (seconds to minutes), whereas type 2 inhibitors take much longer to trap out of the Asp-Phe-Gly-out conformation and display tight binding kinetics that result in a long half life on the enzyme (6–24 h) (Liu and Gray, 2006).

Similar to other small molecule inhibitors, the lack of kinase selectivity of sorafenib, which in a competitive binding assay bound 10% of the 384 kinases tested within 10-fold of that for Raf kinases (Karaman et al., 2008), makes it challenging to pinpoint Raf kinases as the molecular mechanism of 4EBP1 modulation. Therefore, to confirm as well as to identify which Raf isoform is responsible for MAPK regulation of 4EBP1 phosphoprotein levels, QTRRE cells were transfected with B-Raf and Raf-1 siRNA for 72 and 96 h. Real-time PCR analysis of both Raf isoforms, following siRNA treatment, revealed an ~95% decrease in Raf mRNA levels (data not shown). Western blot analysis of Raf-1 siRNA knockdown revealed significant decreases in p-4EBP1-Ser65 and -Thr70 (Fig. 7A), whereas B-Raf siRNA did not affect any p-4EBP1 species (Fig. 7B). Both Raf siRNA’s were target specific, and neither Raf siRNA produced a significant change in total 4EBP1 or GAPDH protein expression.

[FIG. 6. Inhibition of Raf and MEK kinases results in a decrease in p-4EBP1-Ser65, -Thr70, and -Thr37/46 in QTRRE cells. (A) QTRRE cells were treated with sorafenib (50 μM) for 5, 15, 25, 45, 60, and 90 min. (B) QTRRE cells were treated with PD 98059 (50 μM) for 5, 25, 40, and 60 min. 4EBP1 and p-4EBP1-Ser65, -Thr70, and -Thr37/46 were visualized by Western blot analysis. GAPDH was used as a loading control.

FIG. 7. Raf-1 is the dominant regulator of p-4EBP1 protein levels in QTRRE cells. QTRRE cells were transfected with either (A) Raf-1 (100nM) or (B) B-Raf (100nM) ON_TARGETplus SMARTpool siRNA or siCONTROL Non-Targeting siRNA #5 (Dharmacon RNA Technologies, NV). The siRNA-DharmaFECT complex solution was incubated with cells for 72 or 96 h. 4EBP1 and p-4EBP1-Ser65, -Thr70, and -Thr37/46 were visualized by Western blot analysis. GAPDH was used as a loading control. Values are means ± SD (n = 3). A significant difference was seen between control siRNA and Raf-1 or B-Raf siRNA-treated cells at *p < 0.05.
Raf-1 Regulates Cyclin D1 Protein Levels

QTRRE cells, which are null for tuberin, express high levels of p-ERK1/2 and cyclin D1 as well as elevated ERK activity, all of which are reduced following restoration of tuberin expression (Yoon et al., 2004). To investigate whether Raf and MEK1/2 kinases contribute to high cyclin D1 protein levels in QTRRE cells, cyclin D1 was measured by Western blotting following treatment of cells with the Raf kinase inhibitor, sorafenib (50μM), or the MEK1/2 kinase inhibitor PD 98059 (50μM). There was a time-dependent disappearance in cyclin D1 protein subsequent to treatment with either of the inhibitors. Cells treated with sorafenib experienced a 50% reduction in cyclin D1 protein level by 45 min (Fig. 8A). Continued reductions in cyclin D1 protein by sorafenib were observed at 90 min (Fig. 8A). Similarly, treatment with PD 98059 resulted in a 50% reduction in cyclin D1 protein level by 25 min (Fig. 8B) with maximal reduction at 60 min (Fig. 8B). Either sorafenib or PD 98059 treatment resulted in an immediate (5 min) decrease in p-ERK1/2 (Fig. 8). Treatment with sorafenib resulted in a sustained decrease in p-ERK1/2 over a 5- to 60-min period (Fig. 8A); in contrast, suppression of p-ERK1/2 by PD 98059 was reversed between 25 and 60 min (Fig. 8B). The decrease in p-ERK following the treatment with each inhibitor (Fig. 8) correlated with the changes in metabolic activity measured in the MTS assay (Fig. 5). There was little change in total ERK1/2 or GAPDH protein expression following treatment of cells with either inhibitor (Fig. 8), indicative of selective drug action on metabolic function in the presence of sustained cell viability (Fig. 5).

To identify which Raf isoform contributes to MAPK regulation of cyclin D1 protein levels, QTRRE cells were transfected with B-Raf or Raf-1 siRNA for 72 and 96 h. Western blot analysis of Raf-1 siRNA knockdown resulted in a maximal 60% decrease in cyclin D1 protein levels by 96-h posttransfection (Fig. 9A); whereas QTRRE cells treated with B-Raf siRNA experienced a maximal 20% reduction in cyclin D1 by 72 h (Fig. 9B). Neither of the Raf siRNA treatments resulted in a change in p-ERK or total ERK (Cohen et al., 2011). Because both Raf isoforms are activated, as identified in kinase assays (Cohen et al., 2011), when one Raf isoform is knocked down by siRNA, other Rafs can compensate to activate ERK1/2. Both Raf siRNA’s were target specific, and neither Raf siRNA produced a significant change in GAPDH protein expression.

Cyclin D1 mRNA Remains Unchanged Following MAPK Inhibition

The expression of cyclin D1 mRNA was not affected by sorafenib (Fig. 10A) or PD 98059 (Fig. 10B) treatment, indicating that neither sorafenib nor PD 98059 transcriptionally regulated cyclin D1 expression.

Tuberin Negatively Regulates p4EBP1 and Cyclin D1 in Human Kidney Cells.

To determine whether loss of tuberin in human kidney (HK2) cells causes changes in cell signaling comparable to that observed in tumorigenic QTRRE cells, siRNA knockdown of
Tsc-2 was carried out. Tsc-2 siRNA knockdown resulted in an increase in cyclin D1 and in the nonconstitutive higher molecular species of p-4EBP1-Ser65, -Thr70, and -Thr37/46 protein levels (Fig. 11). Tuberin siRNA did not produce a significant change in GAPDH protein expression.

**DISCUSSION**

We describe a novel pathway by which 4EBP1 phosphorylation is modulated by the Raf-1/MEK/ERK MAPK cascade (Figs. 6 and 7). In our tuberin null kidney tumors and tumorigenic QTRRE cells, we found that (1) renal tumors express high protein levels of B-Raf, Raf-1, p-ERK (Fig. 3), 4EBP1, p-4EBP1-Ser65, -Thr70, and -Thr37/46 (Fig. 4) and cyclin D1 (Fig. 1); (2) QTRRE cells express constitutively phosphorylated Raf-1, B-Raf, ERK1/2 (Yoon et al., 2004), and p-4EBP1 (Fig. 6); (3) treatment with sorafenib (Raf inhibitor) and PD 98059 (MEK inhibitor) in QTRRE cells resulted in dephosphorylation of 4EBP1 at Thr65, Thr70, and Thr37/46 (Fig. 6), and consequently, a decrease in cyclin D1 protein levels (Fig. 8) but no change in cyclin D1 mRNA levels (Fig. 10); and (4) Raf-1 and B-Raf siRNA knockdown in QTRRE cells elucidated Raf-1 as the dominant Raf responsible for downstream modulation of 4EBP1 phosphorylation (Fig. 7), which subsequently regulates cyclin D1 translation (Fig. 9).

Downstream signaling from ERK mediates gene expression, metabolism, and cytoskeletal rearrangements, and dysregulation of the pathway alters normal cell growth, differentiation, senescence, and survival (Wellbrock et al., 2004). Hyperactivation of the ERK pathway occurs in over 30% of cancer patients (Wellbrock et al., 2004). Consistent with the high occurrence of ERK activity in a number of cancers, renal tumors derived from Tsc\(^{-}\)EK/\(^{+}\) rats treated for 8 months with TGHQ express elevated protein levels of B-Raf, Raf-1, and p-ERK (Fig. 3) as well as increased kinase activity (Yoon et al., 2002). Furthermore, malignantly transformed QTRRE cells, null for tuberin, also display increased Raf-1, B-Raf, and

**FIG. 9.** Raf-1 is the dominant regulator of cyclin D1 protein levels in QTRRE cells. QTRRE cells were transfected with 100nM (A) B-Raf or (B) Raf-1 ON_TARGETplus SMARTpool siRNA or siCONTROL Non-Targeting siRNA #5 (Dharmacon RNA Technologies, NY). The siRNA-DharmaFECT complex solution was incubated with cells for 72 or 96 h. Whole cell lysates were subjected to Western blotting with antibodies specific to B-Raf, Raf-1, cyclin D1, and GAPDH. Values represent the mean ± SD (n = 3). A significant difference was seen between control siRNA and Raf-1 or B-Raf siRNA-treated cells at *p < 0.05.

**FIG. 10.** Inhibition of Raf and MEK kinases has no effect on cyclin D1 mRNA levels in QTRRE cells. QTRRE cells were treated with (A) sorafenib (50 μM) or (B) PD 98059 (50μM) for 5, 25, 60, and 90 min. PCR products were resolved by agarose gel electrophoresis. n = 3 samples per treatment group, representative PCR product for each group is displayed.
ERK kinase activity (Yoon et al., 2004). Thus, ERK hyper-activation in RCC is associated with loss of tuberin expression, suggesting that ERK regulation lies downstream of tuberin. In accordance with the established tuberin/Rheb/mTOR/4EBP1/cyclin D1 signaling cascade, QTRRE cells and renal tumors express constitutively high protein levels of p-4EBP1-Ser65, -Thr70, and -Thr37/46 (Figs. 4 and 6) and cyclin D1 (Fig. 1). Increased expression of phosphorylated 4EBP1 was recently linked to high-grade prostate, breast, and ovarian tumors (Castellvi et al., 2006; Kremer et al., 2006; Rojo et al., 2007); and the results of this study provide compelling evidence that p-4EBP1 is also prevalent in RCC (Fig. 4).

Inhibition of Raf and MEK with sorafenib or PD 98059, decreased ERK activity and resulted in a decrease in 4EBP1 phosphorylation at sites that are necessary for displacing eIF4E-4EBP1 protein interactions (Fig. 6). These data suggest that ERK1/2 can directly regulate the hierarchical phosphorylation of 4EBP1 downstream of the tumor suppressor tuberin. The order of 4EBP1 phosphorylation is hierarchical, with phosphorylation of Thr37/46 occurring first, followed by Thr70 phosphorylation, and Ser65 last (Gingras et al., 2001). Although amino acids 51–67 on 4EBP1 encompass the eIF4E binding site, all four phosphorylation sites (Thr37/46, Thr70, and Ser65) are necessary for effectively releasing eIF4E from 4EBP1.

Treatment with either Raf or MEK kinase inhibitors appears to decrease phosphorylation on all four putative 4EBP1 phosphorylation sites as well as produce the rapid appearance of a lower molecular weight band (Fig. 6). Interestingly, in combination with the appearance of a lower molecular weight species, there was a substantial increase, most notably with sorafenib treatment, in all three variants of total 4EBP1 (Fig. 6). Similar results on total 4EBP1 accumulation were observed in mouse hepatocytes following in vivo rapamycin treatment, where treatment resulted in an increase in abundance of the lowest molecular weight species (faster migrating) but no decrease in the band intensity of the two higher molecular weight species (slower migration) (Nelsen et al., 2003). This accumulation of total 4EBP1 is likely due to an increase in protein stability because the resultant hypophosphorylation of 4EBP1 would facilitate its subsequent binding to eIF4E, thus decreasing translation initiation. The readily apparent shift in molecular weight between the three species of 4EBP1 is probably due to changes in numerous posttranslational modifications (PTM), beyond the four key sites with known functional significance. 4EBP1 is a highly modified protein, with 17 PTM already identified, most with unknown biological relevance (Cantin et al., 2008; Dephoure et al., 2008; Gevaert et al., 2003; Imami et al., 2008; Matsuoka et al., 2007; Molina et al., 2007).

The hyperphosphorylation of 4EBP1 results in an increased translation of cyclin D1 mRNA due to the complex and dynamic disassociation of 4EBP1 from eIF4E, thus facilitating the ability of eIF4E to complex with its binding partners and initiate cap-dependent translation of cyclin D1 (Gingras et al., 1999, 2001; Jiang and Liu, 2008). Subsequent to the inhibition of Raf and MEK kinases (Fig. 6), the resulting decrease in phosphorylation of 4EBP1-Ser65, -Thr70, and -Thr37/46 led to a consequent decrease in cyclin D1 protein levels (Fig. 8). Treatment of cells with B-Raf and Raf-1 siRNA pinpoints Raf-1 as the dominant Raf regulating MAPK-driven phosphorylation of 4EBP1 (Fig. 7) and the consequent decrease in cyclin D1 (Fig. 9). Previous studies, in a variety of cell types, have correlated sustained ERK activation with the induction of cyclin D1 mRNA (Balmanno and Cook, 1999; Lavoie et al., 1999; Page et al., 1999; Talarmin et al., 1999; Villanueva et al., 2007; Watanabe et al., 1996; Weber et al., 1997). A 6.5-fold increase in cyclin D1 gene expression occurs in kidney tumors derived from TGHQ-treated Tsc-2<sup>−/−</sup> rats (Patel et al., 2003). Interestingly, in the present study, treatment with sorafenib or PD 98059 did not result in a decrease in cyclin D1 mRNA in QTRRE cells (Fig. 10). Studies performed in breast cancer cell lines revealed that ERK5 can activate cyclin D1 transcription (Mulloy et al.,
Following a decrease in all forms of p4EBP1, there is an apparent reduction in phosphorylation at Thr-37 and Ser-46 (Fig. 12 ) because both mTOR and MAPK pathway crosstalk with 4EBP1. Restoration of tuberin antagonizes ras-dependent activation of ERK1 and ERK2 by LPA and EGF (1996). Rapamycin blocks the phosphorylation of 4E-BP1 and inhibits cap-dependent initiation of translation. (1994). Phosphorylation of PHAS-I by mitogen-activated protein (MAP) kinase results in a decrease of p-ERK1/2 and downstream dysregulation of p-4EBP1. Therefore, these results are consistent with previous findings demonstrating that inhibition of mTOR with rapamycin causes a decrease in p4EBP1 and a subsequent decrease in cyclin D1 protein levels, in the absence of changes in cyclin D1 mRNA (Beretta et al., 1996; Beugnet et al., 2003; Gingras et al., 1999, 2001; Hidalgo and Rowinsky, 2000; Nelsen et al., 2003). Thus, we have elucidated a novel mechanism, whereby ERK hyperactivation is responsible for increased translation of cyclin D1.

In summary, our data suggest that inhibition of either the MAPK or mTOR pathway alone is insufficient to abrogate 4E-BP1 phosphorylation (Fig. 12) because both mTOR and ERK are capable of modulating phosphorylation of 4E-BP1 in renal proximal tubule cells. Accordingly, treatment with sorafenib in combination with an analog of rapamycin may be more efficacious than either single agent alone.


