Regulation of B-Raf Kinase Activity by Tuberin and Rheb Is Mammalian Target of Rapamycin (mTOR)-independent*

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Magdalena Karbowniczek‡, Timothy Cash‡, Mitchell Cheung§, Gavin P. Robertson§, Aristotelis Astrinidis‡, and Elizabeth Petri Henske††

From the ‡Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111 and the †Pharmacology Department, The Pennsylvania State College of Medicine, Hershey, Pennsylvania 17033

Tuberous sclerosis complex (TSC) is a tumor suppressor gene syndrome with manifestations that can include seizures, mental retardation, autism, and tumors in the brain, retina, kidney, heart, and skin. The products of the TSC1 and TSC2 genes, hamartin and tuberin, respectively, heterodimerize and inhibit the mammalian target of rapamycin (mTOR). We found that tuberin expression increases p42/44 MAPK phosphorylation and B-Raf kinase activity. Short interfering RNA down-regulation of tuberin decreased the p42/44 MAPK phosphorylation and B-Raf activity. Expression of Rho, the target of the GTPase-activating domain of tuberin, inhibited wild-type B-Raf kinase but not activated forms of B-Raf. The interaction of endogenous Rho with Rho was enhanced by serum and by Ras overexpression. A farnesylation-defective mutant of Rho co-immunoprecipitated with and inhibited B-Raf but did not activate ribosomal protein S6 kinase, indicating that farnesylation is not required for B-Raf inhibition by Rho and that B-Raf inhibition and S6 kinase activation are separable activities of Rho. Consistent with this, inhibition of B-Raf and p42/44 MAPK by Rho was resistant to rapamycin in contrast to Rho activation of S6 kinase, which is rapamycin-sensitive. Taken together these data demonstrate that inhibition of B-Raf kinase via Rho is an mTOR-independent function of tuberin.

The ELT3 cell line (a gift of Dr. Cheryl Walker) was derived from an Eker rat uterine leiomyoma and lacks tuberin expression (34). From ELT3 cells, we previously developed the T3 cell line with stable expression of TSC2 and the V3 vector control-expressing cell line (13). For transient expression, ELT3 cells were retrovirally transduced with GFP as a control, full-length human TSC2, or the N14643 mutant form of TSC2. For retroviral production, GP-293 cells were co-transfected with the respective retroviral construct in the pMSCVneo vector (Clontech) and pVSV-G (Clontech) encoding for the viral glycoprotein using LipofectAMINE 2000 (Invitrogen). Replication-deficient retroviruses were collected from the GP-293 cells at day 3 post-transfection. The transfection efficiency was about 60% as estimated by GFP fluorescence. Where indicated, cells were treated with 20 nM rapamycin (Biomol Research Laboratories, Plymouth Meeting, PA). The Rho cDNA was purchased from Open Biosystems (Huntsville, AL). Rho mutants Q64L and C182S and B-Raf kinase mutants V599E (T1796A), and inactive B-Raf (T598A/S601A) were generated by site-directed mutagenesis using QuikChange site-directed mutagenesis (Stratagene, La Jolla, CA), and sequences

cell cycle (6–10), steroid hormone regulation (11), and Rho activation (12, 13). Hyperphosphorylation of p70 S6 kinase (S6K) and/or its substrate ribosomal protein S6 occurs in cells lacking hamartin or tuberin (14–18). Regulation of S6K by the tuberin-hamartin complex is mediated by the mammalian target of rapamycin (mTOR) (9, 10, 19–21). Tuberin has a highly conserved domain near the carboxyl terminus with homology to Rap1 GTPase-activating protein (GAP) (5, 22). Most recently, Rheb (Ras homolog enriched in brain), a member of the Ras/Rap/Ral subfamily of Ras proteins (23), was established as the direct downstream target of the GAP domain of tuberin (24–30).

While characterizing the effects of tuberin re-expression in tuberin-null ELT3 cells, we found that tuberin expression increases the phosphorylation of p42/44 MAPK. Rho has been shown previously to negatively regulate an upstream activator of p42/44 MAPK, B-Raf kinase (31). However, other investigators have not found a consistent relationship between tuberin expression and p42/44 MAPK activation (27, 29, 32), and one group found that tuberin negatively regulated B-Raf kinase activity (33), the opposite of what would be expected if Rho inhibits B-Raf. Here we demonstrate that tuberin expression increases B-Raf kinase activity and that Rho expression inhibits B-Raf kinase activity. We also found that Rho farnesylation, which is required for activation of mTOR by Rho, is not required for inhibition of B-Raf by Rho. Importantly inhibition of B-Raf by Rho is mTOR-independent unlike activation of S6K by Rho. The mTOR independence of inhibition of B-Raf kinase by Rho has potential therapeutic implications for TSC patients in whom clinical trials using the mTOR inhibitor rapamycin are underway.

EXPERIMENTAL PROCEDURES

Cells, Constructs, siRNA, and Antibodies—The ELT3 cell line (a gift of Dr. Cheryl Walker) was derived from an Eker rat uterine leiomyoma and lacks tuberin expression (34). From ELT3 cells, we previously developed the T3 cell line with stable expression of TSC2 and the V3 vector control-expressing cell line (13). For transient expression, ELT3 cells were retrovirally transduced with GFP as a control, full-length human TSC2, or the N14643 mutant form of TSC2. For retroviral production, GP-293 cells were co-transfected with the respective retroviral construct in the pMSCVneo vector (Clontech) and pVSV-G (Clontech) encoding for the viral glycoprotein using LipofectAMINE 2000 (Invitrogen). Replication-deficient retroviruses were collected from the culture after 72 h and applied to subconfluent ELT3 cells in the presence of 8 μg/ml Polybrene (Sigma). The transfection efficiency was about 60% as estimated by GFP fluorescence. Where indicated, cells were treated with 20 nM rapamycin (Biomol Research Laboratories, Plymouth Meeting, PA). The Rho cDNA was purchased from Open Biosystems (Huntsville, AL). Rho mutants Q64L and C182S and double mutant Q64L/C182S and B-Raf kinase mutants V599E (T1796A), and inactive B-Raf (T598A/S601A) were generated by site-directed mutagenesis using QuikChange site-directed mutagenesis (Stratagene, La Jolla, CA), and sequences

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†To whom correspondence should be addressed: Fox Chase Cancer Center, 333 Cottman Ave., Philadelphia, PA 19111 Tel.: 215-728-2428; Fax: 215-214-1623; E-mail: EP_Henske@fccc.edu.

‡The abbreviations used are: TSC, tuberous sclerosis complex; GAP, homology to Rap1 GTPase-activating protein (GAP) (5, 22). Tuberin has been established as the direct downstream target of the GAP domain of tuberin (12, 13). Hyperphosphorylation of p70 S6 kinase (S6K) and/or its substrate ribosomal protein S6 occurs in cells lacking hamartin or tuberin (14–18). Regulation of S6K by the tuberin-hamartin complex is mediated by the mammalian target of rapamycin (mTOR) (9, 10, 19–21). Tuberin has a highly conserved domain near the carboxyl terminus with homology to Rap1 GTPase-activating protein (GAP) (5, 22). Most recently, Rheb (Ras homolog enriched in brain), a member of the Ras/Rap/Ral subfamily of Ras proteins (23), was established as the direct downstream target of the GAP domain of tuberin (24–30).

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Tuberin Activates B-Raf and p42/44 MAPK

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**RESULTS**

**Tuberin Positively Regulates the Phosphorylation of p42/44 MAPK**—We found that V3 control cells have a low level of phosphorylation of p42/44 MAPK in serum deprivation conditions compared with the T3 tuberin-expressing cell line (Fig. 1A). Similar differences in the phosphorylation of p42/44 MAPK occurred in cells grown in serum (Fig. 1B). To confirm that these phosphorylation differences were not the result of clonal selection, TSC2 was transiently transduced in the parental ELT3 cell line. Increased p42/44 MAPK phosphorylation was again present in tuberin-expressing cells compared with GFP control transduction (Fig. 1A, right panel).

**Tuberin Expression Increases GDP-Rheb**—We confirmed that tuberin regulates the activation state of endogenous Rheb in serum-deprived V3 and T3 cells by measuring the amount of immunoprecipitated Rheb in the GDP-bound state (31, 35). The fraction of GDP-bound immunoprecipitated Rheb in V3 cells was significantly lower (0.64 ± 0.02 fmoI/mg of protein) compared with tuberin-expressing T3 cells (1.08 ± 0.09 fmoI/mg of protein) (Fig. 2A). The fraction of GDP-bound endogenous Rheb was also significantly higher in ELT3 cells with transient expression of TSC2 compared with GFP control (Fig. 2A, right panel).

**Tuberin Increases B-Raf Kinase Activity**—Rheb was previously shown to inhibit B-Raf kinase (31). Therefore, we asked whether B-Raf kinase activity was higher in tuberin-expressing cells compared with tuberin-deficient cells. Endogenous B-Raf kinase was immunoprecipitated from V3 and T3 cells, and its activity was measured using a coupled assay with myelin basic protein as the final substrate. Endogenous B-Raf kinase activity was higher in T3 tuberin-expressing cells compared with V3 cells (Fig. 2B). We next determined whether tuberin containing the patient-derived GAP domain mutation N1643K is defective in GAP activity toward Rheb. Rheb was loaded with [γ-32P]GTP and incubated with immunoprecipitated wild-type tuberin or N1643K tuberin. Tuberin reduced the GTP-Rheb level by about 40% after 15 min and 50% after 30 min (Fig. 2C), while a similar amount of immunoprecipitated N1643K tuberin did not decrease the Rheb-GTP levels. These data are consistent with the results of Garami et al. (29), who found that the expression of N1643K tuberin in COS7 cells did not increase Rheb-GDP levels. Our GAP assay was performed using only immunoprecipitated tuberin. In contrast, Tee et al. (27) incubated Rheb with immunoprecipitated tuberin and hamartin and found that hamartin substantially enhances the GAP activity of tuberin.

In ELT3 cells, transient expression of N1643K tuberin did not increase B-Raf kinase activity (Fig. 2D). Furthermore expression of Rheb in HEK293 cells inhibited endogenous B-Raf kinase activity (Fig. 2E), confirming the previous work of Im et al. (31), and expression of tuberin and hamartin attenuated inhibition of B-Raf by Rheb (Fig. 2E). Taken together, these data indicate that Rheb mediates activation of B-Raf by tuberin.

**Down-regulation of Tuberin with siRNA Decreases B-Raf Kinase Activity**—To verify that tuberin regulates the activity of B-Raf at endogenous expression levels, tuberin was down-regulated in HEK293 cells using siRNA. Tuberin down-regulation decreased the phosphorylation of p42/44 MAPK and decreased the activity of endogenous B-Raf (Fig. 3) consistent with the increase in phospho-MAPK and B-Raf kinase activity with tuberin expression in Figs. 1 and 2. The phosphorylation of ribosomal protein S6 was increased when tuberin was down-regulated as expected.

**Rheb Inhibits the B-Raf-induced Phosphorylation of p42/44 MAPK**—Expression of Rheb in cells expressing endogenous levels of B-Raf had no consistent effect on the phosphorylation of endogenous p42/44 MAPK (not shown). However, when wild-type B-Raf kinase was overexpressed, co-expression of Rheb decreased both the activity of B-Raf kinase and the B-Raf-induced phosphorylation of p42/44 MAPK (Fig. 4A). When serum-deprived cells were stimulated for 15 min with 20% fetal bovine serum, inhibition of p42/44 MAPK phosphorylation by Rheb was much less pronounced (Fig. 4B). In contrast, when cells were grown in serum-starved conditions or continuously in serum, Rheb strongly inhibited the phosphorylation of...
**Fig. 2. Tuberin positively regulates endogenous B-Raf kinase activity.** A, endogenous Rheb was immunoprecipitated from V3 and T3 cells or from cells transduced with GFP or TSC2. GDP-bound Rheb was measured by conversion of GDP to $\gamma^{32}$P-GTP using nucleoside 5'-diphosphate kinase and $\gamma^{32}$P-ATP. The amount of GDP-Rheb was higher in T3 than in V3 cells and in cells expressing TSC2 compared with GFP. Similar results were seen in three independent experiments, each done in duplicate. Rheb expression is shown by immunoblot. *, $p < 0.05$. B, endogenous B-Raf was immunoprecipitated from serum-starved V3 and T3 cells. B-Raf kinase activity was determined in a coupled assay using myelin basic protein as substrate.
Rheb Q64L (39), which is predicted to have constitutive activity based on homology to the constitutively active Ras Q61L mutation; Rheb C182S, which is farnesylation-deficient; and Rheb Q64L/C182S, which is predicted to be dominantly inhibitory based on homology to Ras (40). As expected, expression of Q64L Rheb alone increased the phosphorylation of S6 and 4E-BP (28, 30). Co-expression of Q64L Rheb and B-Raf decreased the B-Raf-induced phosphorylation of p42/44 MAPK by 80% (Fig. 5A). The farnesylation mutant C182S migrated slightly above the other forms of Rheb as seen by other groups (27, 41). Expression of C182S Rheb alone did not increase the phosphorylation of S6, confirming the work of Tee et al. (27) and indicating that farnesylation is required for Rheb to activate mTOR. Importantly, however, C182S Rheb blocked the B-Raf-induced phosphorylation of MAPK by 60%, indicating that farnesylation of Rheb is not required for B-Raf inhibition. Expression of the Q64L/C182S Rheb also decreased the phosphorylation of p42/44 MAPK but did not activate the phosphorylation of S6, again consistent with the hypothesis that farnesylation is required for mTOR activation but not for B-Raf inhibition. The fact that the Q64L/C182S did not function as a dominant negative distinguishes Rheb from Ras for which constitutive active farnesylation mutants act as dominant negatives (40).

**Rheb Farnesylation Is Not Required for Interaction with B-Raf**—An interaction between B-Raf and Rheb has been shown previously by *in vitro* binding studies (42) and by co-immunoprecipitation (31). To determine whether farnesylation is required for interaction of B-Raf with Rheb, we co-expressed Myc-tagged wild-type Rheb and wild-type B-Raf. As shown in Fig. 5B, Myc-Rheb co-immunoprecipitated with B-Raf, and B-Raf co-immunoprecipitated with Myc-Rheb, confirming the earlier studies. We found that the farnesylation-deficient forms of Rheb (C182S Rheb and Q64L/C182S Rheb) also co-immunoprecipitated with B-Raf, indicating that Rheb does not require farnesylation for its interaction with B-Raf.

**The Interaction of Endogenous Rheb and B-Raf Is Enhanced by Serum and by Ras Expression**—We also found that endogenous B-Raf co-immunoprecipitates with endogenous Rheb (Fig. 5C), further supporting a model in which inhibition of B-Raf by Rheb is direct. The endogenous interaction was enhanced ~2-fold in serum-stimulated cells (Fig. 5C) and in cells in which wild-type H-Ras was overexpressed (Fig. 5D). This suggests that the interaction between B-Raf and Rheb is regulated by growth factor signaling pathways. The increased interaction between B-Raf and Rheb when Ras was overexpressed could be linked to the ability of Rheb to inhibit Ras-induced transformation of NIH3T3 cells (39).

We were not able to detect Rheb in B-Raf immunoprecipitates at endogenous expression levels under basal, serum-stimulated, or H-Ras overexpression conditions using either the Upstate Biotechnology B-Raf antibody (07-453), which was generated against amino acids 754–765, or the Santa Cruz Biotechnology B-Raf antibody (SC-5284). The Santa Cruz Biotechnology antibody was substantially more efficient for B-Raf protein as substrate. The activity of immunoprecipitated B-Raf kinase was lower in V3 cells than in tuberin-expressing T3 cells. Data are representative of two independent experiments, each done in duplicate. The expression of total B-Raf kinase is shown by immunoblot. *, *p < 0.05. C, immunoprecipitated wild-type or N1643K mutant tuberin and recombinant GST-Rheb loaded with [γ- ^32^P]GTP were incubated for 15 or 30 min. The fraction of [γ- ^32^P]GTP remaining on Rheb was determined by scintillation counting. Wild-type tuberin reduced the remaining GTP-Rheb by about 40% after 15 min and 50% after 30 min. N1643K tuberin did not reduce the GTP-Rheb levels. Similar results were seen in two independent experiments. The amounts of immunoprecipitated wild-type and N1643K tuberin were similar as shown. *, *p < 0.05 compared with intrinsic activity. D, endogenous B-Raf kinase was immunoprecipitated from ELT3 cells with transient retroviral transduction of GFP, wild-type TSC2, or N1643K/TSC2. Expression of wild-type tuberin, but not N1643K tuberin, increased B-Raf kinase activity. Data represent three independent experiments, each done in duplicate. *, *p < 0.05 relative to GFP control; E, endogenous B-Raf was immunoprecipitated from HEK293 cells expressing empty vector (pcDNA), Rho, or Rho plus tuberin and hamartin. Rheb decreased B-Raf kinase activity. Expression of tuberin and hamartin attenuated inhibition of B-Raf kinase activity by Rheb. *, *p < 0.05 relative to pcDNA; **, **p < 0.05 relative to Rheb alone. IP; immunoprecipitation.
Fig. 4. Rheb inhibits the B-Raf-induced phosphorylation of p42/44 MAPK and the in vitro B-Raf kinase activity. A, B-Raf kinase was expressed in HEK293 cells. Co-expression of Rheb inhibited the phosphorylation of p42/44 MAPK in the cell lysate. B-Raf was immunoprecipitated, and its activity was measured using MEK1 as substrate. Rheb expression decreased the activity of B-Raf. B, B-Raf and Rheb were expressed in HEK293 cells. Rheb inhibited the phosphorylation of p42/44 MAPK in serum-starved cells. After 15 min of stimulation by 20% fetal bovine serum (FBS), inhibition of p42/44 MAPK phosphorylation by Rheb was diminished. C, B-Raf and Rheb were expressed in HEK293 cells. Rheb inhibited the phosphorylation of p42/44 MAPK in serum-starved cells as well as in cells growing continuously in 10% fetal bovine serum. D, co-expression of Rheb and wild-type B-Raf inhibited the B-Raf-induced phosphorylation of p42/44 MAPK by about 60% as expected (lanes 3 and 4). Co-expression of Rheb and either of two active forms of B-Raf (T598E/S601D in lane 5 and V599E in lane 6) did not inhibit the phosphorylation of p42/44 MAPK. As expected, a kinase-dead B-Raf (T598A/S601A) did not activate p42/44 MAPK. Wt, wild-type.

**DISCUSSION**

Here we report that tuberin increases the activity of B-Raf kinase and the phosphorylation of p42/44 MAPK, while Rheb decreases the activity of B-Raf kinase and the phosphorylation of p42/44 MAPK. We observed these effects in tuberin-deficient ELT3 cells in which tuberin was re-expressed and saw reciprocal effects in HEK293 cells in which tuberin was downregulated with siRNA.

In contrast to our data, other groups have not observed effects of tuberin expression on p42/44 MAPK and have concluded that Rheb does not regulate p42/44 MAPK signaling (27, 29). We suspect that this apparent discrepancy is in part the consequence of measuring downstream targets of B-Raf kinase that are also downstream of C-Raf/Raf-1 rather than B-Raf kinase activity itself. Garami et al. (29) found that Rheb expression did not inhibit serum activation of overexpressed p44 MAPK, and Tee et al. (27) found that Rheb expression did not inhibit epidermal growth factor-induced activation of overexpressed ribosomal S6K1, a downstream target of p42/44 MAPK. In HEK293 cells, despite the fact that we consistently detected activation of endogenous B-Raf kinase by tuberin and inhibition of endogenous B-Raf kinase by Rheb, we did not detect differences in the phosphorylation of p42/44 MAPK unless B-Raf kinase was overexpressed. In addition, the inhibitory effects of Rheb on B-Raf as detected using p42/44 MAPK or other downstream targets as a readout may be difficult to detect after serum or epidermal growth factor stimulation when p42/44 MAPK is strongly activated by C-Raf/Raf-1. Consistent with this notion, we found that the inhibitory effects of Rheb on the phosphorylation of p42/44 MAPK were greatly diminished when cells were stimulated with serum. Interestingly, the inhibitory effect of Rheb on p42/44 MAPK was detected in cells grown in the continuous presence of serum. A final factor potentially influencing the conflicting data regarding activation of B-Raf by tuberin is that mouse embryonic fibroblasts are known to express extremely low levels of B-Raf and high levels of A-Raf and C-Raf/Raf-1 (43, 44). This probably accounts for the inability of Zhang et al. (32) to detect differences in the phosphorylation of MAPK in TSC2-null mouse embryonic fibroblasts compared with control mouse embryonic fibroblasts. None of these factors would account for the discrepancy between our data and that of Yoon et al. (33), who found...
that tuberin re-expression decreased B-Raf activity in rat renal epithelial cell lines lacking tuberin. This could indicate that the impact of tuberin on B-Raf kinase is affected by other pathways mutated by quinol-thioether in these cells.

We found that inhibition of B-Raf kinase by Rheb is mTOR-independent. At least one other mTOR-independent function of tuberin has been identified: in mammalian cells, tuberin regulates vascular endothelial growth factor through both mTOR-dependent and mTOR-independent mechanisms (45). Whether or not B-Raf mediates mTOR-independent regulation of vascu-

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**Fig. 5. Interaction of Rheb with B-Raf does not require farnesylation and is enhanced by serum stimulation and Ras expression.**

A, HEK293 cells were transfected with B-Raf and the three mutant forms of Rheb (Q64L, C182S, and C182S/Q64L). The two farnesylation mutants, C182S and C182S/Q64L, migrated slightly above Q64L Rheb. Only Q64L Rheb, and not the farnesylation mutants, increased the phosphorylation of S6K and S6. All three mutants inhibited p42/44 MAPK phosphorylation. B, wild-type Rheb or the mutant forms of Rheb were co-expressed with B-Raf kinase in HEK293 cells. B-Raf was immunoprecipitated using anti-B-Raf antibody (second set of panels), and Myc-Rheb was immunoprecipitated using anti-Myc antibody (third set of panels) followed by Western blot analysis using antibodies to B-Raf and Myc, as indicated. B-Raf co-immunoprecipitated with each form of Rheb, including both farnesylation mutants. Similarly each form of Rheb co-immunoprecipitated with B-Raf. Immunoprecipitation using IgG instead of the primary antibody is shown as a control.

C, endogenous B-Raf co-immunoprecipitated with endogenous Rheb in HEK293 cells. This interaction was enhanced by 2-fold in cells stimulated with 20% fetal bovine serum for 15 min. A similar result was seen in a second independent experiment. D, expression of wild-type H-Ras in HEK293 cells increased by 2-fold the interaction between endogenous Rheb and B-Raf kinase. IP, immunoprecipitation; FBS, fetal bovine serum; Wt, wild-type.
Tuberin Activates B-Raf and p42/44 MAPK

A

\[\begin{array}{c}
\text{Wt-B-Raf:} & + & + & + & + \\
\text{Myc-Wt-Rheb:} & - & - & - & - \\
\text{Rapamycin:} & - & - & - & - \\
\text{B-Raf} & + & + & + & + \\
\text{Myc-Rheb} & - & - & - & - \\
\text{Phospho-p42/44 MAPK} & + & + & + & + \\
\text{Phospho-S6K} & + & + & + & + \\
\text{Phospho-S6} & + & + & + & + \\
\text{B-Raf} & + & + & + & + \\
\text{\(^{32}\text{P}-\text{MEK1}\)} & + & + & + & + \\
\text{IP: B-Raf} & + & + & + & + \\
\end{array}\]

**Fig. 6.** Inhibition of B-Raf kinase by Rheb is mTOR-independent.

A, HEK293 cells were transfected with wild-type B-Raf and Rheb and treated with rapamycin for 30 min. As expected, rapamycin inhibited the Rheb-induced phosphorylation of S6K and S6 (lane 3). In contrast, rapamycin did not release the inhibition of p42/44 MAPK phosphorylation by Rheb (lane 4) or the inhibition of B-Raf kinase activity by Rheb. B, our data place Rheb downstream of tuberin, inhibiting B-Raf and activating mTOR. B-Raf activates mTOR and inhibits B-Raf kinase in an mTOR-independent way. Activation of B-Raf kinase increases the phosphorylation of p42/44 MAPK through MEK. Tuberin deficiency decreases the S6 phosphorylation and decreased p42/44 MAPK phosphorylation. eIF: eukaryotic initiation factor; IP: immunoprecipitation; Wt: wild-type.

myelolipomas, which occur in most TSC patients, also have enlarged, abnormal blood vessels.

An important distinction between signaling of Rheb to B-Raf and mTOR is the differential requirements for Rheb farnesylation. Rheb is known to be farnesylated (39). We found, as did Tee et al. (27), that the farnesyl mutant C182S Rheb was unable to activate S6K. Interestingly, however, C182S retains the ability to interact with B-Raf and inhibit p42/44 MAPK phosphorylation, indicating that farnesylation is not required for Rheb to inhibit p42/44 MAPK. This finding suggests that farnesyltransferase inhibition, a potential therapeutic strategy for TSC patients (27), will not block all consequences of Rheb activation and further confirms that the effects of Rheb on mTOR/S6K and B-Raf/p42/44 MAPK are separable.

Some of the clinical manifestations of TSC are associated with aberrant cellular differentiation, which could be the result of low levels of B-Raf activity in cells lacking tuberin. Renal angiomyolipomas contain vessels, smooth muscle cells, and fat. All three components arise from a common precursor cell indicating differentiation plasticity (17, 47). The neurons within cerebral cortical tubers in TSC also exhibit aberrant cellular differentiation (48). B-Raf is required for the neuronal differentiation of PC12 cells, suggesting another possible link between B-Raf signaling and the clinical manifestations of TSC.

In conclusion, our data demonstrate for the first time that tuberin activates B-Raf and p42/44 MAPK, that inhibition of p42/44 MAPK by Rheb does not require farnesylation, and that inhibition of B-Raf and p42/44 MAPK by Rheb is mTOR-independent. The fact that cells lacking tuberin have decreased B-Raf kinase activity and decreased phosphorylation of p42/44 MAPK is somewhat surprising given the role of tuberin as a tumor suppressor gene and the role of B-Raf in positively regulating cell proliferation and differentiation. We hypothesize that dysregulated B-Raf kinase activity due to tuberin deficiency could lead to the aberrant cellular differentiation seen within some TSC tumors. In addition, altered neuronal differentiation could be linked to the neurologic manifestations of TSC, which include mental retardation and autism. The mTOR-independent functions of tuberin are of particular interest clinically because rapamycin is currently in clinical trials to determine its potential as a therapy for the renal and pulmonary manifestations of TSC.

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