Rheb Inhibits C-Raf Activity and B-Raf/C-Raf Heterodimerization*

Received for publication, June 1, 2006, and in revised form, June 23, 2006 Published, JBC Papers in Press, June 27, 2006, DOI 10.1074/jbc.M605273200

Magdalena Karbowniczek1, Gavin P. Robertson1, and Elizabeth Petri Henske2,3

From the 1Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111 and the 2Pharmacology Department, Pennsylvania State College of Medicine, Hershey, Pennsylvania 17033

The Ras-Raf-MEK signaling cascade is critical for normal development and is activated in many forms of cancer. We have recently shown that B-Raf kinase interacts with and is inhibited by Rheb, the target of the GTPase-activating domain of the tuberous sclerosis complex 2 gene product tuberin. Here, we demonstrate for the first time that activation of Rheb is associated with decreased B-Raf and C-Raf phosphorylation at residues Ser-446 and Ser-338, respectively, concomitant with a decrease in the activities of both kinases and decreased heterodimerization of B-Raf and C-Raf. Importantly, the impact of Rheb on B-Raf/C-Raf heterodimerization and kinase activity are rapamycin-insensitive, indicating that they are independent of Rheb activation of the mammalian target of rapamycin-Raptor complex. In addition, we found that Rheb inhibits the association of B-Raf with H-Ras. Taken together, these results support a central role of Rheb in the regulation of the Ras/B-Raf/C-Raf/MEK signaling network.

B-Raf, A-Raf, and C-Raf (also called Raf-1) are members of the Raf kinase family, which regulate the activation of the MEK2 (MAPK/ERK-activating kinase)/MAPK signaling cascade and share Ras as a common activator. B-Raf and C-Raf heterodimerize, thereby increasing the activity of both kinases (1, 2). Both B-Raf and C-Raf mutations are associated with human cancers (3–5). B-Raf mutations, which occur in melanoma, thyroid, and other cancers and in developmental disorders, including cardio-facio-cutaneous syndrome (6–8), are more common than C-Raf mutations. However, because of the heterodimerization of C-Raf and B-Raf, which impacts the activity of both kinases (1, 2), mutations in one kinase can influence the activity of the other. A small molecule inhibitor of Raf was approved in 2006 for the treatment of renal cancer (9, 10). We and others have previously shown that Rheb (Ras homolog enriched in brain) inhibits B-Raf kinase activity (11, 12). Rheb, a member of the Ras/Rap/Ral subfamily of Ras proteins (13), is inhibited by the GTPase-activating domain of the tuberous sclerosis complex 2 (TSC2) gene product, tuberin (14–20). Activation of Rheb also results in activation of the mammalian target of rapamycin (mTOR) (14–19, 20), resulting in translational initiation and cell growth. Rheb inhibition of B-Raf kinase is insensitive to rapamycin (12), which is a specific inhibitor of the mTOR-Raptor complex (21–23). Therefore, Rheb appears to have two separate functions: mTOR activation and B-Raf inhibition. However, whether Rheb influences the activity of other members of the Raf kinase family is not known. It is also not known whether Rheb inhibition of B-Raf impacts the association of B-Raf with Ras and C-Raf.

We demonstrate here that Rheb activation inhibits the kinase activity of C-Raf and strongly inhibits the heterodimerization of B-Raf and C-Raf. We also found that Rheb inhibits the association between B-Raf and H-Ras. Our data position Rheb as a key regulator of the Ras/B-Raf/C-Raf/MEK signaling cascade, with implications for the pathogenesis of TSC and for the design of targeted strategies to inhibit Raf kinases.

EXPERIMENTAL PROCEDURES

Cells, Constructs, siRNA, and Antibodies—HEK293 cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen). The Tsc2(−/−) p53−/− and Tsc2(+/+) p53−/− MEFs were the gifts of Dr. David Kwiatkowski (Dept. of Medicine, Brigham and Women’s Hospital and Harvard Medical School, Boston, MA). When indicated, cells were serum-deprived for 18 h. Transfections were performed using Lipofectamine 2000 (Invitrogen). Where indicated, cells were treated for 24 h with 20 nm rapamycin (Biomol Research Laboratories, Plymouth Meeting, PA). The B-Raf kinase mutants were generated by site-directed mutagenesis using QuikChange site-directed mutagenesis (Stratagene, La Jolla, CA) and fully sequence-confirmed. The C-Raf cDNA and the C-Raf mutant S338D/Y341D/T491E/S494D (DEDD) were the gifts of Dr. Kun-Liang Guan (Life Sciences Institute, Dept. of Biological Chemistry, Institute of Gerontology, University of Michigan, Ann Arbor, MI). For siRNA down-regulation of TSC2 or Rheb, cells were transfected with 100 nm of either TSC2 SMARTpool siRNA or Rheb SMARTpool siRNA or nonspecific control siRNA (both from Dharmacon, Lafayette, CO) using Trans-IT TKO transfection reagent (Mirus, Madison WI). The following antibodies were used: anti-tuberin C20, anti-B-Raf (F-7), anti-C-Raf (E10), and anti-H-Ras (Santa Cruz Biotechnology, Santa Cruz, CA); anti-phospho-Ser-235/236 S6 ribosomal protein, anti-Myc, anti-HA, and anti-phospho-Ser-217/222 MEK1, anti-phospho-Thr-202/Tyr-204-p42/44 MAPK (Cell Signaling, Beverly, MA); anti-phos-
Rheb Inhibits C-Raf Kinase

**FIGURE 1.** Rheb decreases C-Raf phosphorylation at Ser-338 and kinase activity in a rapamycin-resistant manner. 

**A** endogenous C-Raf was immunoprecipitated from serum-starved or serum-stimulated HEK293 cells. Rheb expression decreased C-Raf phosphorylation at Ser-338 by 3-fold after 10 min of serum stimulation. Rapamycin had no effect on Rheb inhibition of C-Raf phosphorylation. 

**B** the activity of immunoprecipitated C-Raf was measured using MEK1 as substrate. Co-expression of Rheb inhibited C-Raf kinase activity and Ser-338 phosphorylation in the presence of serum by 2-fold. 24 h of rapamycin treatment did not block Rheb inhibition of C-Raf activity. The results of three independent experiments were normalized and compared (*, p < 0.05 by Student’s paired t test). 

**C** wild-type and DDED mutant C-Raf were expressed in HEK 293 cells. The in vitro kinase activity of C-Raf-DDED was not inhibited by co-expression of Q64L Rheb. 

**D** tuberin down-regulation using siRNA decreased the activity of C-Raf in the presence of serum, using MEK1 as substrate and detected using either the phospho-MEK1 antibody or 32P-labeled MEK1 (upper panel). The results of three independent experiments were normalized and compared. 

**E** Rheb down-regulation using siRNA increased the phosphorylation of endogenous C-Raf at Ser-338, in both serum-starved and serum-stimulated conditions (upper panel). We achieved ~70% of Rheb down-regulation at the mRNA level compared with control siRNA, as determined by real-time PCR (data not shown). The results of three independent experiments were normalized and compared.
FIGURE 1—continued

**C**

| Condition                        | +  | +  | -  | -  | -  | -  | +  | +  | -  | -  |
|----------------------------------|----|----|----|----|----|----|----|----|----|----|
| Flag-C-Raf                      |    |    |    |    |    |    |    |    |    |    |
| Flag-C-Raf-DDDE                 | -  | -  | +  | +  | +  | +  | -  | +  | +  | +  |
| Myc-Q64L-Rheb                   | -  | +  | -  | +  | +  | -  | +  | -  | -  | +  |
| 10 min, 20% FBS                 | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  |

**In vitro kinase assay**

Flag-C-Raf

Myc-Rheb

Whole cell lysate

**D**

| Condition                        | +  | -  | +  | -  | +  | +  | -  | -  | +  | +  |
|----------------------------------|----|----|----|----|----|----|----|----|----|----|
| Control siRNA                    |    |    |    |    |    |    |    |    |    |    |
| TSC2 siRNA                       | -  | +  | -  | +  | +  | +  |
| 10 min, 20% FBS                  | -  | -  | +  | +  | +  | +  |

**Phospho-MEK1-Ser 217/222**

**In vitro kinase assay**

32P-MEK1

C-Raf

Tuberin

Whole cell lysate

**E**

| Condition                        | +  | -  | +  | -  | +  | +  | -  | +  | -  | +  |
|----------------------------------|----|----|----|----|----|----|----|----|----|----|
| Control siRNA                    |    |    |    |    |    |    |    |    |    |    |
| Rheb siRNA                       | -  | +  | -  | +  | +  | +  |
| 10 min, 20% FBS                  | -  | -  | +  | +  | +  | +  |

**Phospho-Ser 338**

IP: C-Raf

Rheb

β-actin

Phospho-S6

Whole cell lysate

**Fold Ser338 phosphorylation**

p=0.03

FIGURE 1—continued
Rheb Inhibits C-Raf Kinase

Rheb Inhibits C-Raf Kinase activity and is required for serum-induced C-Raf activation (24–27). We expressed tagged C-Raf and Rheb in HEK293 cells and immunoprecipitated either Myc-Rheb or FLAG-C-Raf, under both serum starvation and serum stimulation conditions. We were not able to detect an interaction between Rheb and C-Raf (Fig. 2A), in contrast to the interaction between Rheb and B-Raf, which was demonstrated by our group (12) and others (11). These data suggest that Rheb inhibition of C-Raf involves an indirect mechanism and led us to ask whether Rheb-dependant inhibition of C-Raf could be mediated via B-Raf, as C-Raf and B-Raf form functional heterodimers upon Ras or serum stimulation (1, 2, 29), which synergistically enhances the activity of both kinases (1, 29, 30). We hypothesized, therefore, that Rheb inhibition of C-Raf might be the consequence of decreased B-Raf/C-Raf heterodimerization. To test this, we immunoprecipitated endogenous B-Raf from cells transfected with C-Raf. Rheb expression strongly inhibited the association of C-Raf with endogenous B-Raf, in both serum-starved and serum-stimulated cells (Fig. 2B). Rheb inhibition of the B-Raf/C-Raf association was not blocked by 24 h of rapamycin treatment, indicating that it is mTOR-Raptor independent (Fig. 2B). To confirm these results, we immunoprecipitated endogenous C-Raf from cells transfected with B-Raf alone or with Rheb. Rheb expression almost completely blocked B-Raf association with endogenous C-Raf, and again this was rapamycin-insensitive (Fig. 2C). These results, together with the lack of evidence of co-immunoprecipitation of Rheb and C-Raf, suggest that C-Raf inhibition by Rheb is a consequence of decreased B-Raf activity and B-Raf/C-Raf heterodimerization, thereby preventing full activation of C-Raf.

The V600E mutation in B-Raf is present in the majority of malignant melanomas and in some ovarian, colorectal, and thyroid cancers (4, 31). V600E has a basal activity ~10-fold higher than wild-type B-Raf (4). Previously, we demonstrated that Rheb does not inhibit V600E B-Raf kinase activity (12). Here, we asked whether Rheb can inhibit the association of V600E B-Raf with C-Raf. We expressed either
FIGURE 2. Rheb inhibits the heterodimerization of B-Raf and C-Raf. A, wild-type Rheb was co-expressed with C-Raf kinase in HEK293 cells. C-Raf was immunoprecipitated using anti-FLAG antibody, and Myc-Rheb was immunoprecipitated using anti-Myc antibody, and followed by Western blot analysis using antibodies to FLAG and Myc, as indicated. C-Raf did not co-immunoprecipitate with Rheb, and Rheb did not co-immunoprecipitate with C-Raf, in either serum-starved or serum-stimulated cells. B, C-Raf was co-expressed with Rheb or vector control in HEK293 cells, and endogenous B-Raf or C-Raf was immunoprecipitated. 24 h of rapamycin treatment did not block the Rheb-induced inhibition of B-Raf/C-Raf heterodimerization. C, B-Raf was co-expressed with either Rheb or vector control in HEK293 cells, and B-Raf and C-Raf was immunoprecipitated. Rheb expression strongly inhibited B-Raf association with C-Raf, compared with the vector control, both in serum-starved cells and serum-stimulated cells. 24 h of rapamycin treatment did not block the Rheb-induced inhibition of B-Raf/C-Raf heterodimerization. D, wild-type B-Raf or V600E mutant B-Raf was expressed in HEK293 cells with or without Rheb, and C-Raf was immunoprecipitated. The association of both wild-type and V600E B-Raf with C-Raf was markedly reduced when Rheb was expressed, compared with the vector control.
FIGURE 3. Rheb negatively regulates the phosphorylation of B-Raf at S446. A, the Ser-446 phosphorylation and *in vitro* kinase activity of immunoprecipitated endogenous B-Raf were decreased after siRNA down-regulation of tuberin in HEK293 cells, compared with control siRNA, in serum starvation, continuous growth in serum, and in cells stimulated for 15 min with hepatocyte growth factor. An increase in the phosphorylation of ribosomal protein S6 was seen after tuberin down-regulation, reflecting Rheb activation of mTOR. B, B-Raf and Rheb were expressed in HEK293 cells. Rheb inhibited B-Raf phosphorylation on Ser-446 in serum-starved cells, as well as in serum-starved cells stimulated for 15 min with platelet-derived growth factor (25 ng/ml), hepatocyte growth factor (50 ng/ml), or 30 min with epidermal growth factor (25 ng/ml). The results of four independent experiments were normalized and compared (*, p < 0.05 by Student’s paired t test). C, wild-type B-Raf or V600E mutant B-Raf was expressed in HEK293 cells with or without Rheb and B-Raf was immunoprecipitated. The Ser-446 phosphorylation of both wild-type and V600E B-Raf was markedly reduced when Rheb was expressed compared with the vector control. D, expression of Rheb inhibited the activity of wild-type B-Raf, as measured using MEK1 as substrate. Rheb did not inhibit the activity of V600E B-Raf. E, wild-type, S446D and T599E/S602D B-Raf mutants were expressed in HEK 293 cells. The *in vitro* kinase activity of wild-type and Ser-446 mutant form of B-Raf (S446D), but not T599E/S602D, were inhibited by co-expression of Rheb, in both serum starvation (*left panel*) and serum stimulation (*right panel*) conditions. The results of three independent experiments were normalized and compared.
wild-type B-Raf or V600E B-Raf alone or with Rheb and immunoprecipitated endogenous C-Raf. Rheb expression strongly inhibited the interaction of endogenous C-Raf with V600E B-Raf in both serum deprivation and serum stimulation conditions (Fig. 2D).

Rheb Negatively Regulates the Phosphorylation of Ser-446 on B-Raf Kinase —To verify that loss of endogenous tuberin (and consequent activation of endogenous Rheb) is associated with decreased B-Raf activity, we used siRNA to down-regulate endogenous tuberin (TSC2) in HEK293 cells. As expected, tuberin down-regulation decreased the in vitro kinase activity of endogenous B-Raf (Fig. 3A). Interestingly, siRNA down-regulation of tuberin inhibited Ser-446 phosphorylation of endogenous B-Raf (Fig. 3A). Phosphorylation of Ser-446 is correlated with B-Raf activity (24, 32), and Pak 1 has been identified as a kinase for this site (32). To determine whether Rheb inhibition of Ser-446 B-Raf phosphorylation is growth factor-specific, HEK293 cells were serum-starved overnight and then treated for 15 min with platelet-derived growth factor or hepatocyte growth factor or for 30 min with epidermal growth factor. Rheb inhibition of Ser-446 phosphorylation was evident in all three conditions (Fig. 3B). Rheb also inhibited the Ser-446 phosphorylation of V600E B-Raf (Fig. 3C). However, the in vitro kinase activity of V600E B-Raf was not significantly inhibited by Rheb expression (Fig. 3D), suggesting that Ser-446 phosphorylation is not the mechanism through which Rheb inhibits B-Raf. To directly test this, we generated a S446D (phospho-mimetic) mutant form of B-Raf. The in vitro kinase activity of the S446D B-Raf mutant was strongly inhibited by Rheb (Fig. 3E), indicating that Rheb inhibition of B-Raf Ser-446 phos-
Rheb inhibits Ras association with B-Raf. A, the in vitro kinase activity toward MEK1 of immunoprecipitated endogenous B-Raf was decreased in Tsc2\(^{-/-}\) p53\(^{-/-}\) MEFs compared with Tsc2\(^{+/+}\) p53\(^{-/-}\) MEFs. B, Tsc2\(^{-/-}\) p53\(^{-/-}\) MEFs were transfected with control or Rheb siRNA. Endogenous B-Raf was immunoprecipitated, and the immunoprecipitates were blotted with anti-H-Ras antibody. B-Raf association with H-Ras was significantly increased when Rheb expression was down-regulated, compared with control siRNA. C, HA-tagged B-Raf was co-expressed with Rheb or vector control in HEK293 cells. Endogenous H-Ras was immunoprecipitated, and the immunoprecipitate was immunoblotted with anti-HA antibody. B-Raf association with H-Ras was markedly reduced when Rheb was co-expressed, compared with the vector control. D, S446A, S446D, and wild-type B-Raf were expressed in HEK293 cells. Endogenous H-Ras was immunoprecipitated, and the immunoprecipitates were immuno-blotted with anti-HA antibody. B-Raf association with H-Ras was inhibited when the S446A B-Raf mutant was expressed in serum-starved conditions. In serum-stimulated cells (right panel), there was no effect of the S446A mutant on the association of C-Raf and B-Raf. E, HA-tagged S446A and S446D B-Raf were co-expressed with Rheb in HEK293 cells. Endogenous H-Ras was immunoprecipitated, and the immunoprecipitates were immunoblotted with anti-HA antibody. S446A B-Raf association with H-Ras was inhibited, as expected (see Fig. 4D). Rheb expression did not inhibit the association between S446D B-Raf and H-Ras.

Serum starvation and serum stimulation conditions (Fig. 4A). We then asked whether Rheb activation in the Tsc2\(^{-/-}\) MEFs impacts the association of Ras with B-Raf by using siRNA to down-regulate Rheb and immunoprecipitating endogenous B-Raf. Rheb down-regulation in TSC2\(^{-/-}\) MEFs significantly enhanced the binding of endogenous H-Ras and B-Raf (Fig. 4B). To confirm this finding, we expressed B-Raf and the hyperactive Q64L Rheb in HEK293 cells and immunoprecipitated endogenous H-Ras. Rheb very strongly inhibited the association of endogenous H-Ras with B-Raf in both serum starvation and serum stimulation conditions (Fig. 4C).

Ras Binding to B-Raf Is S446-dependent—Because we had found that Rheb inhibits both B-Raf Ser-446 phosphorylation (Fig. 3, A and B) and B-Raf association with H-Ras (Fig. 4, B and C), we next asked whether B-Raf Ser-446 phosphorylation influences the B-Raf/H-Ras interaction. We generated S446A (non-phosphorylatable) and S446D (phospho-mimetic) forms of B-Raf and expressed them with B-Raf in HEK293 cells. The S446A B-Raf bound substantially less H-Ras than the S446D or wild-type B-Raf (Fig. 4D), suggesting that Ser-446 phosphorylation regulates the interaction between B-Raf and Ras. Furthermore, whereas Rheb expression disrupted the interaction between wild-type B-Raf and H-Ras (Fig. 4C), Rheb did not disrupt the association between S446D B-Raf and H-Ras (Fig. 4E). These results suggest that Ser-446 phosphorylation of B-Raf promotes the association of B-Raf and Ras, possibly due to conformational changes and/or release of B-Raf autoinhibition.

Taken together, our data lead to a model (Fig. 5) in which Rheb directly interacts with and inhibits B-Raf kinase. This direct interaction inhibits C-Raf/B-Raf heterodimerization, with consequent decrease in C-Raf activity, and decreases Ser-446 B-Raf phosphorylation, resulting in inhibition of the B-Raf/H-Ras interaction.

DISCUSSION

The Ras/B-Raf/C-Raf signaling network plays an essential role in normal development and is activated in many forms of cancer. Rheb is known to inhibit the activity of B-Raf kinase, but whether Rheb has a broader impact on this network has not

phorylation is independent of Rheb inhibition of B-Raf kinase activity. Rheb also inhibited a double phospho-mimetic mutant form of B-Raf, S446D/S447D (data not shown). These findings indicate that inhibition of Ser-446 phosphorylation is not the mechanism through which Rheb inhibits B-Raf, and are instead consistent with a model in which Rheb directly inhibits B-Raf kinase, leading to inhibition of phosphorylation (see Fig. 5). Rheb did not inhibit a constitutively active form of B-Raf, T599E/S602D (33) (Fig. 3E).

Rheb Inhibits Ras Binding to B-Raf—We next turned our attention to the interaction between B-Raf and Ras. B-Raf in vitro kinase activity is enhanced by the addition of Ras (34), and activated Ras stimulates the heterodimerization of B-Raf and C-Raf (1). Therefore, we asked whether Rheb influences Ras binding to B-Raf. First, we confirmed that B-Raf is inhibited in Tsc2\(^{-/-}\) MEFs, in which endogenous Rheb is activated. The in vitro kinase activity of endogenous B-Raf kinase was inhibited in TSC2\(^{-/-}\) MEFs, compared with Tsc2\(^{+/+}\) MEFs, in both

Serum starvation and serum stimulation conditions (Fig. 4A). We then asked whether Rheb activation in the Tsc2\(^{-/-}\) MEFs impacts the association of Ras with B-Raf by using siRNA to down-regulate Rheb and immunoprecipitating endogenous B-Raf. Rheb down-regulation in TSC2\(^{-/-}\) MEFs significantly enhanced the binding of endogenous H-Ras and B-Raf (Fig. 4B). To confirm this finding, we expressed B-Raf and the hyperactive Q64L Rheb in HEK293 cells and immunoprecipitated endogenous H-Ras. Rheb very strongly inhibited the association of endogenous H-Ras with B-Raf in both serum starvation and serum stimulation conditions (Fig. 4C).

Ras Binding to B-Raf Is S446-dependent—Because we had found that Rheb inhibits both B-Raf Ser-446 phosphorylation (Fig. 3, A and B) and B-Raf association with H-Ras (Fig. 4, B and C), we next asked whether B-Raf Ser-446 phosphorylation influences the B-Raf/H-Ras interaction. We generated S446A (non-phosphorylatable) and S446D (phospho-mimetic) forms of B-Raf and expressed them with B-Raf in HEK293 cells. The S446A B-Raf bound substantially less H-Ras than the S446D or wild-type B-Raf (Fig. 4D), suggesting that Ser-446 phosphorylation regulates the interaction between B-Raf and Ras. Furthermore, whereas Rheb expression disrupted the interaction between wild-type B-Raf and H-Ras (Fig. 4C), Rheb did not disrupt the association between S446D B-Raf and H-Ras (Fig. 4E). These results suggest that Ser-446 phosphorylation of B-Raf promotes the association of B-Raf and Ras, possibly due to conformational changes and/or release of B-Raf autoinhibition.

Taken together, our data lead to a model (Fig. 5) in which Rheb directly interacts with and inhibits B-Raf kinase. This direct interaction inhibits C-Raf/B-Raf heterodimerization, with consequent decrease in C-Raf activity, and decreases Ser-446 B-Raf phosphorylation, resulting in inhibition of the B-Raf/H-Ras interaction.

DISCUSSION

The Ras/B-Raf/C-Raf signaling network plays an essential role in normal development and is activated in many forms of cancer. Rheb is known to inhibit the activity of B-Raf kinase, but whether Rheb has a broader impact on this network has not
been previously addressed. Here, we report that Rheb inhibits the activity of C-Raf kinase, the heterodimerization of B-Raf and C-Raf, and the interaction of B-Raf with H-Ras.

We found that Rheb decreases C-Raf activity and C-Raf Ser-338 phosphorylation, a site that is strongly linked to C-Raf activity. However, Rheb did not completely block Ser-338 phosphorylation, suggesting that Rheb-independent factors are also involved. In contrast to the B-Raf-Rheb interaction that is evident at endogenous expression levels (12), we did not detect an interaction between C-Raf and Rheb in vivo. This suggests that Rheb inhibition of C-Raf is indirect, potentially mediated via Rheb inhibition of B-Raf/C-Raf heterodimerization. Heterodimerization of B-Raf and C-Raf is recognized as a significant mechanism of Raf kinase regulation (1, 2). Epidermal growth factor does stimulate C-Raf in B-Raf-deficient fibroblasts (29), and depletion of B-Raf inhibits C-Raf activity (35). It is hypothesized that this intricate signaling network, in which B-Raf modifies Ser phosphorylation through C-Raf, provides subtle regulation of signaling intensity or duration (2). Our data are consistent with a model in which Rheb inhibition of C-Raf kinase activity is the result of decreased B-Raf activity and subsequent decreased B-Raf/C-Raf heterodimerization. To our knowledge, Rheb is the first small GTPase shown to inhibit B-Raf/C-Raf heterodimerization. We hypothesize that Rheb binding to B-Raf induces a conformational change in B-Raf, which inhibits S446-B-Raf phosphorylation and subsequent Ras activation and inhibits B-Raf/C-Raf heterodimerization and subsequent C-Raf activity. However, we cannot exclude the possibility that Rheb inhibition of C-Raf is mediated by a Raf phosphatase. Rheb also inhibits the interaction of B-Raf and H-Ras in a B-Raf S446-dependent manner. These data suggest that one consequence of Rheb binding to B-Raf is decreased Ser-446 phosphorylation, resulting in enhanced B-Raf autoinhibition and inhibition of Ras binding.

The clinical consequences of Rheb inhibition of the Ras/B-Raf/C-Raf network in cells carrying mutations in TSC1 or TSC2 is not yet known. Tumors in TSC patients, including renal angiomyolipomas and subependymal giant cell astrocysts exhibit aberrant differentiation patterns (36, 37), which may be mediated by Raf kinases. Abnormal angiogenesis occurs in TSC angiomyolipomas and angiofibromas, which is intriguing because B-Raf-deficient mice have vascular defects (29). B-Raf is required for the neuronal differentiation of PC12 cells (38), so it is also possible that Rheb regulation of Raf kinases contributes to the neurological manifestations of TSC, which can include mental retardation and autism.

Rapamycin is currently being used in clinical trials for TSC patients. Because Rheb inhibition of B-Raf and C-Raf is rapamycin-insensitive, it will be of particular interest to examine the phenotypes of TSC tumors after rapamycin therapy. Finally, it is possible that the two independent growth-related functions of Rheb (B-Raf/C-Raf inhibition and mTOR activation) may naturally limit the ability of Rheb to induce tumor growth, contributing to the fact that the vast majority of tumors in TSC patients are benign.

In conclusion, we found that Rheb inhibits C-Raf, B-Raf/C-Raf heterodimerization, and B-Raf/H-Ras association, revealing a novel mechanism of Raf signaling regulation. The activity of tuberin and Rheb are regulated by growth factor signals, nutrient availability, and energy status. Therefore, our data position Rheb as an integrator of upstream cues to modulate Raf activity. This function of Rheb may be related to the unusual aspects of tumorigenesis in TSC patients, including aberrant vascular development and abnormal cellular differentiation patterns. Raf kinase inhibition is an active area of cancer research, with the Raf inhibitor Sorafenib approved for the treatment of renal cell carcinoma in 2006 (10) and other pharmacological inhibitors in development. Defining the precise role of Rheb in the regulation of Raf kinases could lead to novel approaches for targeted Raf kinase inhibition.

Acknowledgments—We are grateful to Drs. Jon Chernoff, Erica Golemis, and Victoria Robb for their critical reviews of this manuscript.

REFERENCES

1. Weber, C. K., Slupsky, J. R., Kalmes, H. A., and Rapp, U. R. (2001) Cancer Res. 61, 3595–3598
2. Garnett, M. J., Rana, S., Paterson, H., Barford, D., and Marais, R. (2005) Mol. Cell 20, 963–969
3. Wellbrock, C., Karasarides, M., and Marais, R. (2004) Nat. Rev. Mol. Cell Biol. 5, 875–885
4. Davies, H., Bignell, G. R., Cox, C., Stephens, P., Edkins, S., Clegg, S., Teague, J., Wooffendin, H., Garnett, M. J., Bottomley, W., Davis, N., Dicks, E., Ewing, R., Floyd, Y., Gray, K., Hall, S., Hawes, R., Hughes, J., Kosmidou, V., Menzies, A., Mould, C., Parker, A., Stevens, C., Watt, S., Hooper, S., Wilson, R., Jayatilake, H., Gusterson, B. A., Cooper, C., Shipley, J., Hargrave, D., Pritchard-Jones, K., Maitland, N., Chenevix-Trench, G., Riggins, G. J., Bigner, D. D., Palmieri, G., Cosu, A., Planagan, A., Nicholson, A., Ho, J. W., Leung, S. Y., Yuen, S. T., Weber, B. L., Seigler, H. F., Darrow, T. L., Paterson, H., Marais, R., Marshall, C. J., Wooster, R., Stratton, M. R., and Futreal, P. A. (2002) Nature 417, 949–954
5. Zebisch, A., Staber, P. B., Delavar, A., Bodner, C., Hiden, K., Fischereder, K., Janakiraman, M., Linkes, W., Auner, H. W., Emberger, W., Windpassinger, C., Schimek, M. G., Hoeller, G., Tropf, M., and Sill, H. (2006) Cancer Res. 66, 3401–3408
6. Rodriguez-Viciana, P., Tetsu, O., Tidyman, W. E., Estep, A. L., Conger, B. A., Cruz, M. S., McCormick, F., and Rauen, K. A. (2006) Science 311, 1287–1290
7. Schubbert, S., Zenker, M., Rowe, S. L., Boll, S., Klein, C., Bollag, G., van der Burgt, I., Mansante, L., Kalscheuer, V., Wehner, L. E., Nguyen, H., West, B., Zang, K. Y., Sisterman, E., Rauch, A., Niemann, C. M., Shannon, K., and Kratz, C. P. (2006) Nat. Genet. 38, 331–336
8. Niidomi, T., Aoki, Y., Narumi, Y., Neri, G., Cave, H., Verloes, A., Okamoto, N., Hennekam, R. C., Gilleesen-Kaebach, G., Wiezorek, D., Kavamura, M. I., Kurosawa, K., Ohashi, H., Wilson, L., Heron, D., Bonneau, D., Corona, G., Kaname, T., Naritomi, K., Baumann, C., Matsumoto, N., Katoh, K.,...
Rheb Inhibits C-Raf Kinase