Hyperactivation of Mammalian Target of Rapamycin (mTOR) Signaling by a Gain-of-Function Mutant of the Rheb GTPase

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Gain-of-function mutants of Ras and Rho family small GTPases have proven to be important tools in analyzing signaling downstream of these small GTPases. The Ras-related GTPase Rheb has emerged as a key player downstream of TSC1–2 in activating signaling to mammalian target of rapamycin (mTOR) effectors of cell growth such as S6K and 4E-BP1. The TSC1–2 tumor suppressor complex has been shown to act as a RhebGAP, converting Rheb from a GTP-bound to a GDP-bound form. Here we report the identification of a mutant Rheb (S16HRheb) that exhibits gain-of-function properties. At endogenous levels of expression S16HRheb exhibits increased GTP loading in vivo and is resistant to TSC1–2 GAP in vitro. Compared with wild-type Rheb, S16HRheb is more active at promoting the phosphorylation of the mTOR effectors S6K1 and 4E-BP1. Thus S16HRheb will help to identify proximal signaling events downstream of Rheb and allow potential Rheb-independent functions downstream of TSC1–2 to be investigated.

The TSC1–2 tumor suppressor complex acts to negatively regulate mTOR signaling, with loss of function in humans responsible for the benign hamartomatous syndrome, tuberous sclerosis complex, and increased cell number and cell and organ growth in Drosophila (reviewed in Ref. 1). Genetic screens in Drosophila have identified Rheb as a critical regulator of growth, and both genetic and biochemical evidence indicates that TSC1–2 inhibits Rheb and suppresses growth by acting as a RhebGAP (2–4). Both TSC1 and TSC2 appear necessary for RhebGAP activity (5) and a so-called “asparagine thumb” involving Asn^{464} of the TSC2 GAP domain, has been suggested to participate in GTP hydrolysis (6), analogous to the regulation of Rap1 by RapGAP (7). Consistent

with this notion, this residue is mutated in tuberous sclerosis complex (8) and renders TSC1–2 unable to regulate mTOR signaling to S6K1 (9).

Although loss of function of Rheb is epistatic to TSC1–2 loss of function in Drosophila in regulation of growth (4), it is unclear why loss of function of TSC1–2 promotes increased cell number in Drosophila (10), while gain-of-function of Rheb by overexpression does not (4). A possible explanation is that TSC1–2 has targets in addition to Rheb involved in regulating cell number. To help clarify this issue it will be necessary to compare phenotypes of TSC1–2 deficiency with that of Rheb rendered insensitive to TSC1–2 GAP. In K-Ras, gain-of-function mutations in codon 12 have been generated at endogenous levels in conditional mouse models resulting in tumor phenotypes analogous to human cancers in which mutation of these residues are found (11, 12). However, the functional consequences of similar mutations in Rheb are not known. Here we report the analysis of a Rheb mutant (S16HRheb) that exhibits gain-of-function properties similar to that of V12Ras in the activation of mTOR signaling to S6K and 4E-BP1.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—pRK5myc-Rheb and pGEX-4T2-Rheb constructs were generated by reverse transcription-PCR using Rheb-specific primers to clone Rheb from human brain cDNA (Clontech). Mutant Rhebs in pRK5myc or pGEX-4T2 were obtained using a QuikChange site-directed mutagenesis kit (Stratagene). pEGFPN1TSC1 has been described (13); pCMV-FLAG-TSC1 and pCMV-TSC2, pCDNA3HA-4E-BP1, and pRK556K-GST were provided, respectively, by Dr. Julian Sampson, Dr. Nahum Sonenberg, and Dr. George Thomas.

Cell Culture, Transfection, and Immunoblotting—HEK293T cells were transfected with Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s instructions. For experiments with S6K or 4E-BP1 reporter plasmids cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 50 mM NaF, 1 mM NaN3, 1 mM EDTA, 1 mM EGTA, 0.27 M sucrose, 5 mM MgCl2, 0.1% β-mercaptoethanol, and Complete protease inhibitors (Roche Applied Science). For 4E-BP1 experiments blots were fixed in 0.2% glutaraldehyde after transfer. Commercial antibodies were as follows: anti-FLAG M2 (Sigma), anti-phospho-Thr^{389} S6K, anti-HA, and anti-phospho-Ser^{65} 4E-BP1 (Cell Signaling Technology) and anti-S6K1 monoclonal (Transduction Laboratories). Rabbit anti-human Rheb was generated by immunizing rabbits with GST–Rheb and affinity purified with GST–Rheb chemically cross-linked to glutathione-agarose.

In Vivo Rheb Guanine Nucleotide Binding—HEK293T in 10-cm² plates were transfected using Lipofectamine 2000. Forty hours after transfection, cells were washed once with phosphate-free Dulbecco’s modified Eagle’s medium (Invitrogen), incubated with phosphate-free Dulbecco’s modified Eagle’s medium for 2 h, and labeled with 0.1 mCi of [32P]orthophosphate (Amersham Biosciences)/ml for 4 h. Cells harvested as described (14) were centrifuged for 10 min at

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ACCELERATED PUBLICATION: Activated Rheb GTPase

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| H. Sapiens | D. Melanogaster | B. Taurus | A. Fumigatus | C. Familiaris | X. Laevis | S. Pombe | S. Cerevisiae | C. Elegans |
|------------|----------------|----------|--------------|--------------|----------|----------|--------------|-----------|
| H6FQ-------| HP------------| HP-------| MP-----------| HP-----------| MP-------| HP-------| MEYATMS---  | NENGK-------|
| S4KRI-------| AILGLY-------| TRERHK----| AIPAFK-------| RIEGR-------| LKI-------| MIL-------| NSSST---NHF  | QOKR-------|
| W9KVS------| QETOG-------| QETOG----| QETOG-------| QETOG-------| QETOG----| QETOG----| QETOG-------| QETOG------|
| QSITQ-------| QTFEG-------| QTFEG----| QTFEG-------| QTFEG-------| QTFEG----| QTFEG----| QTFEG-------| QTFEG------|
| VDSY--------| PTD------TITT| PTD------  | PTD------TITT| PTD------TITT| PTD------  | PTD------  | PTD------TITT| PTD------TITT|

14,000 rpm at 4 °C and lysates adjusted to 0.5 M NaCl prior to immunoprecipitation with 5 μg 9E10 anti-myc antibody and protein G-Sepharose beads for 2 h. Beads were washed and nucleotides eluted and resolved by TLC as described (14). [32P]GTP and [32P]GDP levels were imaged and quantified on a PhosphorImager using ImageQuant software (Amersham Biosciences).

In Vitro GAP Assays—5 × 10^6 HEK 293T cells were transfected with 20 μg each of pEGFPN1TSC1 and pCMV FLAG TSC2 or with empty vector using Lipofectamine 2000. Cells were incubated for 44 h, the last 24 h being in serum-free medium and lysis and assays performed as described (5). Guanine nucleotides were quantified using ImageQuant software (Amersham Biosciences).

RESULTS AND DISCUSSION

We reasoned that in organisms such as budding yeast Saccharomyces cerevisiae and Caenorhabditis elegans lacking TSC1–2, but encoding orthologues of Rheb, there would be no selective pressure to maintain sensitivity to TSC1–2 GAP activity. Thus residues that are conserved in Rheb from species containing TSC1–2, but divergent in both S. cerevisiae and C. elegans, could potentially render Rheb sensitive to TSC1–2 action. By aligning protein sequences in ClustalW, we identified three residues (serines 16 and 89 and threonine 42 in human Rheb) that are conserved in organisms that contain TSC1–2 but have diverged in both S. cerevisiae and C. elegans (Fig. 1A). Our attention was drawn to one of these positions, Ser^16, for two reasons. First, in Ras this position is equivalent to glycine at codon 13, a residue that is subject to oncogenic mutations that are thought to render Ras resistant to GAP-stimulated hydrolysis (15). Second, this residue is immediately adjacent to arginine 15 (Arg^15), a residue that, when mutated to glycine (R15G), has been reported to render Rheb more highly GTP loaded and partially resistant to TSC1–2 GAP activity in vivo (6). A recent report using PCR-based mutagenesis and genetic screening has also identified three mutations in Schizosaccharomyces pombe Rheb (equivalent to V17G, K120R, and N153S in human Rheb) that potentially exhibit gain-of-function properties (16).

FIGURE 1. Identification and analysis of Rheb mutations. A, alignment of Rheb proteins equivalent to residues 1–108 in human Rheb. Sequences were identified using Entrez and by NCBI Blast and translation searches with human Rheb and aligned in ClustalW with identity shown in red, strong similarity in green, weak similarity in blue, and differences in black. Boxed residues are conserved in all species except S. cerevisiae and C. elegans. GenBank™ accession numbers are as follows: Q15382 (human (Homo sapiens)), NP730950 (Drosophila melanogaster), AA102508 (Bos taurus), XM748989 (Aspergillus fumigatus), XPS2768 (Canis familiaris), AAH43818 (Xenopus laevis), Q94363, (S. pombe), P25378 (S. cerevisiae), and P34443 (C. elegans). B, S6K1-GST reporter assay for activity of Rheb mutants. 250 ng of pRK5myc expressing wild-type Rheb (equivalent to V17G, K120R, and N153S in human Rheb) that are conserved in organisms lacking TSC1–2, but divergent in both S. cerevisiae and C. elegans, could potentially render Rheb sensitive to TSC1–2 action. By aligning protein sequences in ClustalW, we identified three residues (serines 16 and 89 and threonine 42 in human Rheb) that are conserved in organisms that contain TSC1–2 but have diverged in both S. cerevisiae and C. elegans (Fig. 1A). Our attention was drawn to one of these positions, Ser^16, for two reasons. First, in Ras this position is equivalent to glycine at codon 13, a residue that is subject to oncogenic mutations that are thought to render Ras resistant to GAP-stimulated hydrolysis (15). Second, this residue is immediately adjacent to arginine 15 (Arg^15), a residue that, when mutated to glycine (R15G), has been reported to render Rheb more highly GTP loaded and partially resistant to TSC1–2 GAP activity in vivo (6). A recent report using PCR-based mutagenesis and genetic screening has also identified three mutations in Schizosaccharomyces pombe Rheb (equivalent to V17G, K120R, and N153S in human Rheb) that potentially exhibit gain-of-function properties (16). However, whether analogous mutations increase the ability of mammalian Rheb to activate mTOR signaling is not known.

As an initial screen for function and sensitivity to TSC1–2 action therefore we expressed wild-type or Rheb mutated at Arg^15 (R15G), Ser^16 (S16H and S16N), and Ser^89 (S89D, S89N) as well as the equivalent mutations identified in S. pombe (N153S, V17G, K120R) as myc-tagged proteins alone or together with TSC1–2 along with a S6K-GST reporter in HEK293T cells. Overexpression of wild-type Rheb strongly stimulates the phosphorylation of S6K1 at T389, a residue required for S6 kinase activity (Fig. 1b). In the absence of overexpressed TSC1–2, S16H, S16N, and N153S activate S6K more potently than the other mutants or wild-type Rheb. When TSC1–2 is overexpressed we obtain similar results: S16H, S16N, and N153S all remain able to strongly activate S6K activation (S16H > S16N > N153S, see supplemental Fig. S1). However, both R15G and V17G are suppressed by TSC1–2 as
**FIGURE 2.** GTP loading and GTPase activity of wild-type and Rheb mutants. A, in vivo GTP loading. Phosphorimage of a thin layer chromatograph of $^{32}$P-labeled guanine nucleotides eluted from immunoprecipitated myc-Rheb, pRK5myc vector or pRK5mycRheb plasmids transfected in the presence of pCMVTag vector (− TSC1–2) or FLAG-TSC1 and FLAG-TSC2 plasmids (+ TSC1–2). The middle and bottom panels indicate controls for expression of myc-Rheb (bottom panel, arrow) and FLAG-TSC1 (middle panel, arrow) and FLAG-TSC2 (middle panel, arrowhead) performed in a parallel assay performed without $^{32}$P metabolic labeling. B, quantitation of GTP loading. From $n = 3$ experiments, values from the Phosphomager of GDP and GTP intensity were divided by 2 and 3, respectively, to account for $^{32}$P incorporation and percent GTP calculated as $100 \times (\text{GTP} / (\text{GTP} + \text{GDP}))$. The asterisk represents the use of Student’s t test to establish statistical confidence in the difference in percent GTP between wild-type Rheb and S16HRheb in the absence of TSC1–2 ($n = 3, p < 0.02$) or presence of TSC1–2 ($n = 3, p < 0.005$). Coomassie Blue-stained GST-Rheb and GST-S16H Rheb used in GAP assays, along with BSA standards used to calculate protein inputs to assay. D, in vitro GAP assays. Image of a thin layer chromatograph of $^{32}$P-labeled guanine nucleotides eluted from GST-Rheb and S16H Rheb exposed to x-ray film. Wild-type or S16H GST-Rheb loaded with [α-$^{32}$P]GTP were subjected to GAP assays in triplicate for 60 min with FLAG immunoprecipitates from mock-transfected HEK393T (− TSC1–2) to measure intrinsic GTP hydrolysis and from FLAG-TSC2/pEGFPN1TSC1 (+ TSC1–2)-transfected cells to measure GAP-mediated hydrolysis. Assays were performed three times with similar results, and the autoradiograph of a single experiment is shown. E, quantitation of assay shown in D. Percent GTP was calculated from a phosphorimage of the TLC plate by measuring the intensity of GDP and GTP in three assays. The asterisk represents the use of Student’s t test to establish statistical confidence in the difference in percent GTP following intrinsic hydrolysis between wild-type Rheb and S16H Rheb in the absence of TSC1–2 ($n = 3, p < 0.002$).
strongly as wild-type Rheb, while K120R and both S89D and S89N show partial resistance to overexpressed TSC1–2.

To confirm that the increased ability of S16HRheb to activate S6K1 T389 phosphorylation in the presence of TSC1–2 is due to the inability of TSC1–2 to stimulate GTP hydrolysis of this mutant, we examined the in vivo guanine nucleotide loading of wild-type and Rheb mutant proteins. Constructs encoding myc-tagged wild-type Rheb, R15G, S16H, and N153S were transfected alone or together with FLAG-tagged TSC1 and TSC2 into HEK293T cells, which were labeled with 32P, and Rheb proteins immunoprecipitated and bound guanine nucleotides analyzed. The results show that of the three mutants analyzed, the absence of overexpressed TSC1–2–S16HRheb exhibits the highest GTP loading (83% ± 4 for S16H compared with 64% ± 6 for wild-type Rheb, p < 0.02, n = 3). In the presence of TSC1–2 both R15GRheb and N153SRheb exhibit slightly reduced sensitivity to TSC1–2 compared with wild-type Rheb (27 ± 5% for wild-type Rheb, Fig. 2, A and B, p < 0.005, n = 3).

To characterize further the S16H mutation we examined both binding and dissociation of guanine nucleotides. Surprisingly, although more highly GTP loaded in vivo than wild type, S16HRheb exhibits slightly reduced binding to both GDP and GTP and a slightly increased rate of dissociation of both nucleotides (see supplemental Fig. S3). To explore whether instead the mutation affects GTP hydrolysis, we performed in vitro GAP assays with recombinant GST-Rheb and GST-S16HRheb (Fig. 2C). Significantly, the hydrolysis of wild-type GST-Rheb, but not S16HRheb, is strongly stimulated by TSC1–2 GAP activity (wild-type Rheb remaining 2.2 ± 0.2% GTP bound; S16H Rheb 88.8 ± 0.8% GTP bound, Fig. 2, C–E). In addition, S16HRheb exhibits a small reduction in intrinsic GTP hydrolysis compared with wild-type Rheb (89.4 ± 0.4% GTP bound versus 79.8 ± 1.8%, p < 0.002, n = 3). Identical results are obtained using a charcoal-release assay in which the release of [32P]P, is measured (see supplemental Fig. S4). Thus the S16H mutation confers resistance to TSC1–2 GAP activity in vitro.

These results suggest that at endogenous levels S16HRheb may be insensitive to TSC1–2 and therefore behave as a gain-of-function mutant in activating mTOR signaling. To explore this possibility, we performed a titration experiment expressing myc-tagged wild-type or S16HRheb at different levels along with reporter plasmids for S6K1 (S6K1-GST) and 4E-BP1 (HA-4E-BP1) and determined the phosphorylations of S6K1-T389 and 4E-BP1 S65, both of which have been shown to be stimulated by TSC1–2 deficiency and Rheb overexpression (17, 18). The results show that, when expressed at levels close to endogenous Rheb (around 8 ng of transfected plasmid), wild-type Rheb does not promote S6K1 reporter T389 phosphorylation in serum-starved HEK293T cells, while S16HRheb remains able to stimulate T389 phosphorylation (Fig. 3A). For the 4E-BP1 reporter, wild-type Rheb poorly stimulates phosphorylation at Ser65 at the expression levels used (up to 250 ng of plasmid) compared with the S6K1 reporter. However, S16HRheb is still able to strongly promote phosphorylation at this level of expression and at an increased level compared with wild-type Rheb down to as low as 8 ng of plasmid of transfected plasmid (Fig. 3B). Thus when expressed at endogenous levels S16HRheb exhibits gain-of-function properties in promoting the phosphorylation of two downstream effectors of mTOR.

The structures of Rheb complexed to GTP and GDP have recently been determined indicating a structural alteration of the switch 1 effector loop upon GTP binding (19). However, the

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**FIGURE 3. Increased activation of mTOR signaling by RhebS16H.** A and B, titration of pRK5myc wild-type Rheb and S16HRheb with S6K-GST reporter (A) and with 4E-BP1 reporter (B). HEK293T cells were transfected with pRK5myc or various amounts of pRK5myc Rheb in combination with pRK5myc vector and 0.1 g of pCDNA3 HA-4E-BP1 reporter plasmids. Cells were serum-starved for 3 h before lysis and Western blotted with the indicated antibodies. Endogenous Rheb is indicated with an arrowhead and myc-Rheb with an arrow.
structure of Rheb in the transition state of GTP hydrolysis when complexed to TSC1–2 is not known. Thus it is difficult to assess how the Ser16 → His substitution inhibits TSC1–2 action. However, in the structure of H-Ras complexed to GDP, AlF₃ and GAP-334, a mimic of the transition state of Ras during GTP hydrolysis, it is clear that weak interactions occur between the Cα atom of Gly₁² of Ras and the main chain CO of Arg⁷⁸⁹ of GAP. Thus it has been proposed that this mode of interaction renders most mutations at Gly₁² resistant to GAP and therefore oncogenic (20). It is plausible therefore that in the transition state of GTP hydrolysis interactions involving Ser16 of Rheb and N1643 of the TSC2 GAP limit the substitutions at Ser16 that permit efficient hydrolysis of GTP. Interestingly, substitution of Ser16 by glycine has reported not to affect Rheb GTP loading (21), suggesting that the bulky imidazole ring of histidine in this position may be critical in preventing TSC1–2 GAP action. Since S16HRheb activates mTOR signaling to S6K and 4E-BP1 when expressed at low levels, it may prove to be a useful tool in identifying effector mechanisms coupling Rheb.GTP to mTOR signaling and help to identify potential Rheb-independent functions of TSC1–2.

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