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Rheb G-Proteins and the Activation of mTORC1

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Abstract

Rheb belongs to a unique family within the Ras superfamily of G-proteins. Although initially identified in rat brain, this G-protein is highly conserved from yeast to human. While only one Rheb is present in lower eukaryotes, two Rheb proteins exist in mammalian cells. A number of studies establish that one of the functions of Rheb is to activate mTOR leading to growth. In particular, the ability of Rheb to activate mTORC1 *in vitro* points to direct interaction of Rheb with the mTORC1 complex. Additional functions of Rheb that are independent of mTOR have also been suggested.

II. Rheb Defines a Unique Family Within the Ras Superfamily G-Proteins

A. The Ras Superfamily G-Proteins

The Ras superfamily of G-proteins regulates a variety of signal transduction processes [1, 2]. These proteins are monomeric proteins of approximately 20–30 kDa with conserved structures and functions. These proteins bind guanine nucleotides and function as a molecular switch by shuttling between GTP-bound and GDP-bound forms. The Ras superfamily consists of subfamilies of Ras, Rho, Rab, Arf, and Ran with Ras serving as the founding member. The pathways controlled by these proteins include signal transduction, cellular growth, transformation, transport, motility, traffic, and adhesion. Although these proteins have intrinsic GTPase activity, GTP hydrolysis is greatly enhanced by a set of proteins called GAPs (GTPase activator proteins) [3, 4], which maintain these proteins in the inactive GDP-bound state. On the other hand, the exchange of GDP to GTP is mediated by another set of proteins called GEFs (guanine nucleotide exchange factors) [5, 6], which maintain the proteins in the active GTP-bound state. An extra layer of regulation is provided by an additional set of proteins called GDIs (GDP dissociation inhibitors), which inhibit nucleotide exchange by binding to the GDP-bound form [7, 8].

The Ras superfamily is highly conserved across species ranging from yeast to mammals, including *Drosophila*, *C. elegans*, and plants. The proteins contain five conserved G boxes: G1, G2, G3, G4, and G5. A core effector domain called the switch I domain, which is involved in the interaction with downstream effectors, is conserved. Majority but not all of the superfamily members undergo a posttranslational modification at their C-terminus. These modifications facilitate membrane association and proper subcellular localization of the proteins which is critical for their functions. The C-terminus of many proteins end with a

motif known as the CaaX box, which consists of a tetrapeptide sequence (C = Cys, a = aliphatic, X = C-terminal amino acid) [9]. This motif is modified by the addition of two types of lipid chains—farnesyl or geranylgeranyl groups—mediated by protein farnesyltransferase and protein geranylgeranyltransferase I, respectively. Other motifs such as CC, CCX, CCXX are also found to exist and they are modified by protein geranylgeranyltransferase II [10]. A few members have a modification at their N-terminus which involves the addition of a myristate fatty acid.

B. Rheb Family Genes

The *Rheb* gene was first discovered in 1994 by Yamagata *et al.* [11] as a transcript which was rapidly induced in hippocampal granule cells by seizures and by NMDA-dependent synaptic activity. The product of this gene encodes a protein belonging to the Ras superfamily of small G-proteins. Rheb shares a 30–40% sequence identity with the Ras proteins. The Rheb proteins are highly conserved from yeast to mammals but are absent in prokaryotes [12, 13]. Rheb has intrinsic GTPase activity and can alternate between an active GTP-bound form and an inactive GDP-bound form [14].

In yeasts (*S. cerevisiae*, *S. pombe*), Rheb is encoded by a single gene [13]. Similarly, a single *Rheb* gene was identified in *Drosophila* [15]. However, the *Rheb* gene in *Drosophila* is alternatively spliced to yield two isoforms (Isoform A and Isoform B) that differ in their 5' untranslated regions [16]. In mammals, there are two genes designated as *Rheb1* and *Rheb2* (*RhebL1*) [15]. The gene is located on chromosome 3 in *S. cerevisiae*, *C. elegans*, and *Drosophila* and on chromosome 2 in *S. pombe*. In humans, Rheb1 is located on chromosome 7 while Rheb2 is on chromosome 12. In addition, three *Rheb* pseudogenes exist in the human genome—two on chromosome 10 and one on chromosome 22. The genomic structure of human *Rheb1* contains seven exons while that of human *Rheb 2* consists of eight exons. At the protein level, the coding sequences of the two human *Rheb* genes share a 52% identity. *Rheb1* is ubiquitously expressed with high-level expression in skeletal muscle and cardiac muscles. On the other hand, the expression of *Rheb2* is more limited with high expression in brain [17].

C. Rheb Protein Structure

The Rheb protein consists of 209 amino acids in *S. cerevisiae* (ScRheb), 185 amino acids in *S. pombe* (SpRheb), 207 amino acids in *C. elegans* (CeRheb), and 182 amino acids in *Drosophila melanogaster* (DmRheb) [13]. Sequence alignment shows that both ScRheb and CeRheb proteins have an N-terminal extension of 7–10 residues, function of which is not known. In humans, HsRheb1 consists of 184 amino acids while HsRheb2 consists of 183 amino acids and the two proteins share a 51% identity. HsRheb1 shares a 63% identity with DmRheb, 43% identity with CeRheb, 53% identity with SpRheb, and 37% identity with ScRheb. The molecular weight of the Rheb proteins ranges from 20 to 24 kD in the species mentioned above. Structure of Rheb proteins is shown in Figure 3.1.

Like the Ras proteins, the Rheb proteins contain five G boxes involved in GTP binding and hydrolysis [13]. Interestingly, most Rheb contains an arginine residue (R15) at the position equivalent to glycine (G12) in Ras [12, 13]; G12 is highly conserved in all Ras proteins and

mutation to any other residue (except proline) results in the oncogenic transformation of Ras [1]. Significance of the conserved arginine is unclear at the moment. The last four residues at the C-terminus consist of the CaaX motif where the cysteine is farnesylated posttranslationally yielding a mature protein lacking the terminal three amino acids (aaX residues are removed). The CaaX motif in Rheb proteins is -CSIM (ScRheb), -CVIA (SpRheb), -CSIS (CeRheb), -CLVS (DmRheb), -CSVM (HsRheb1), and -CHLM (HsRheb2) while it is -CVLS in the Ras proteins.

The protein structure of HsRheb1 is similar to that of Ras and Rap proteins and consists of six β -sheets and five α -helices [18]. The N-terminal 169 residues constitute the GTPase domain while the 15 residues at the C-terminus are hypervariable and show flexibility. The protein contains two switch regions (Switch I and Switch II) involved in recognition and interaction with GAPs, GEFs, and effectors. On the basis of sequence alignment, it has been observed that the switch I region in Rheb consists of residues 33–41 corresponding to residues 30–38 of Ras, while the switch II region consists of residues 63–79, corresponding to residues 60–76 of Ras. Despite the similarities, there are few interesting variations in the Rheb structure as compared to Ras [18]. The phosphate binding loop (P-loop) found in the G1 domain contains a highly conserved glycine at position 12 (G12) in the Ras family of proteins while in Rheb the equivalent position is occupied by an arginine (R15). A G12R mutation in Ras greatly decreases its intrinsic GTPase activity and renders it resistant to the action of RasGAP. Although wild-type Rheb containing R15 also has very low intrinsic GTPase activity, it is nevertheless sensitive to its GAP (Tsc2). In addition, the orientation of the side chain of R15 in Rheb proteins suggests that it is not directly involved in GTP hydrolysis and thus may not be responsible for Rheb's intrinsic low GTPase activity. Although both proteins have similar switch I regions, there are major differences in the switch II regions. In Ras, the switch II region assumes an α -helical conformation which undergoes a marked change upon GTP/GDP cycling; however, in Rheb the switch II region assumes an unraveled conformation undergoing only minor changes in response to GTP/GDP cycling. In both Ras and Rheb proteins, switch II has a highly conserved glutamine (Q61 and Q64, respectively). Q61 in Ras is directly responsible for GTP hydrolysis, but Q64 in Rheb has not been implicated to contribute to GTP hydrolysis as a result of the unique switch II region in Rheb.

D. Farnesylation and Localization of Rheb

Farnesylation of Rheb proteins has been demonstrated with mammalian Rheb [19, 20] and the importance of farnesylation for the activation of the mTOR signaling has been reported [20–24]. On the other hand, postprenylation processes such as proteolytic cleavage and carboxylmethylation are not required for mTOR activation [22]. Effects of farnesyltransferase inhibitors (FTIs) on the mTOR signaling have been reported [20, 23, 25]. Rheb can produce rapid development of aggressive and drug-resistant lymphomas in mice, and FTI treatment can block Rheb activity and induce antitumor effects in lymphoma cells [26]. Studies in yeast also suggested the significance of farnesylation on the function of Rheb [13, 27]. In *S. pombe*, Rheb is essential, as shown by the induction of cell cycle arrest at the G0/G1 phase when SpRheb is disrupted. Farnesylation of wild-type SpRheb is critical for complementing this phenotype [27]. Genetic screens to identify suppressors of the *tsc*

phenotypes showed that the loss of farnesylation by the mutation in the protein farnesyltransferase β -subunit gene suppresses overactivation of the Tsc/Rheb/TOR signaling [28].

Rheb is localized to endomembranes. Buerger *et al.* [21] used EGFP-Rheb and monitored its localization in HEK293 cells. This study showed that EGFP-Rheb is localized within a defined ring around the nucleus, reminiscent of ER, and Golgi apparatus. The protein was shown to activate mTOR only when it reached the Golgi membrane. This localization was farnesylation dependent, as farnesylation deficient mutants failed to localize to the endomembrane vesicles. An intracellular localization was also observed for Rheb2 protein [29]. Sancak *et al.* [30] also used EGFP-Rheb and showed that Rheb colocalizes with *Discosomared* fluorescent protein-labeled Rab7, suggesting that Rheb is localized to the late endosomal and lysosomal compartments. Saito *et al.* [17] expressed EGFP tagged HsRheb1 and HsRheb2 in MDCK, HeLa, astrocytoma, and HEK293 cells and found that Rheb induced the formation of large vacuoles and that the tagged proteins were localized to the vacuolar membranes. These vacuoles were found to be late endocytic vesicles, as the late endocytic markers (Rab7 and Rab9) colocalized with Rheb. Vacuole formation did not occur when a mutant version of Rheb, lacking the C-terminal CaaX motif, was used whereby a diffuse pattern of localization was observed. Endomembrane localization of Rheb was also reported by Takahashi *et al.* [31]. Another study carried out by Maet *et al.* [32] suggested that HsRheb is localized to the mitochondria, as examined by the colocalization with mitotracker in HeLa cells. More recently, Hanker *et al.* [22] examined localization of GFP-Rheb1 and GFP-Rheb2 in COS-7 cells by comparing with markers for intracellular compartments. This study showed that both Rheb1 and Rheb2 localize to ER and Golgi but not to endosomes, lysosomes, or mitochondria.

E. Biochemical Activity and Regulators

Rheb is a G-protein and can bind both GTP and GDP [11, 14, 15, 33]. Biochemical studies using purified recombinant *Drosophila* Rheb (DmRheb) indicate that Rheb binds GTP preferentially over GDP and that this binding requires Mg^{2+} [15]. The binding is both time and nucleotide concentration dependent. Low but significant GTPase activity was detected with human Rheb as well as with fission yeast Rheb [14, 33]. DmRheb also exhibits low intrinsic GTPase activity [15]. The presence of arginine at the position corresponding to amino acid 12 of Ras does not completely eliminate its GTPase activity. The percentage of GTP bound to Rheb was examined as a measure of activation levels of endogenous Rheb [14]. The analysis showed that Rheb's activation state remained unusually high for a Ras superfamily member (above 20%).

A heterodimer TSC1/TSC2 consisting of the *TSC1* and *TSC2* gene products functions as a GTPase activating protein for Rheb converting GTP-bound form to a GDP-bound form. TSC2 protein contains a region (amino acids 1517–1674) of homology to the catalytic domain of Rap1-GAP at its C-terminal region and a fragment containing this domain exhibits the activity to stimulate intrinsic GTPase activity of Rheb [34]. The ability of TSC1/TSC2 to stimulate intrinsic GTPase activity of Rheb was demonstrated by expressing TSC1 and TSC2 and examining effects on Rheb [23, 24, 35–37]. In these experiments, the ratio of

GTP and GDP bound to Rheb was examined after releasing guanine nucleotides from Rheb, a method described by Li *et al.* [38]. More recently, a novel method based on NMR was reported [39]. This method enables observation of real-time GAP activity. The study confirmed that TSC1/TSC2 accelerated GTP hydrolysis by Rheb approximately 50-fold. This is through an “asparagines-thumb” mechanism that uses a catalytic asparagine instead of arginine finger found in Ras-GAP [34, 39].

In contrast to GAP, no GEF proteins have been identified up to now. The protein TCTP (Translationally Controlled Tumor Protein) has recently been reported to function as the putative GEF for Rheb in *Drosophila* [40]. These findings were corroborated by Dong *et al.* [41], who demonstrated the interaction between human TCTP (HsTCTP) and human Rheb (HsRheb) *in vitro* and activation of the mTORC1 pathway by HsTCTP *in vivo*. A crystal structure of the E12V mutant of HsTCTP revealed that it lacked GEF activity as a result of a loss of interaction with Lys-45 of HsRheb. In addition, in *Arabidopsis thaliana* TCTP was identified as an important regulator of growth implying the plant TCTP as a mediator of TOR activity similar to that observed in nonplant systems [42]. However, contradictory reports have questioned the role of TCTP as the GEF for Rheb. Studies of HsRheb done *in vivo* and *in vitro* by Rehmann *et al.* [43] demonstrated no interaction between human Rheb and TCTP and subsequent findings by Wang *et al.* [44] also suggested that human TCTP does not interact with Rheb and has no influence on mTOR signaling.

Regulation of Rheb function by binding proteins has been reported. Bnip3, a hypoxia-inducible Bcl-2 homology 3 domain-containing protein, directly binds Rheb and inhibits the mTOR signaling [45]. Bnip3 was identified by carrying out yeast two-hybrid screen of cDNA libraries from HeLa cells and mouse embryos using Rheb as a bait. Bnip3 decreases Rheb GTP levels and inhibits the ability of Rheb to induce S6K phosphorylation. Another protein that was reported to bind Rheb is glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [46]. Under low glucose conditions, GAPDH prevents Rheb from binding to mTOR and inhibits mTORC1 signaling.

F. Rheb Mutants

Extensive studies in *S. pombe* led to the identification of dominant-negative mutants of SpRheb, D60V, D60I, and D60K, whose expression led to growth inhibition and G1 arrest [33]. The D60V and D60I mutants preferentially bind GDP, while D60K binds neither GDP nor GTP. The D60I mutant was found to be more potent than D60V as its GTP binding capacity was severely reduced and GDP, once bound to it, could not be displaced by excess GTP. In Ras proteins, D57 is the critical residue involved in GTP/GDP binding. These mutations were introduced into mammalian Rheb to derive dominant negative forms. While they exhibit the ability to downregulate the mTOR signaling [33], expression of these mutants was low limiting their use for the analysis of the mTOR signaling.

Mutational analyses in *S. pombe* also led to the identification of hyperactive mutants of Rheb having point mutations at valine-17, serine-21, lysine-120, or asparagine-153 [47]. Cells expressing these mutants were found to exhibit resistance to canavanine and thialysine. These mutants exhibit an overall increase in the GTP/GDP ratio. Using sucrose gradient sedimentation experiments, it was determined that only the hyperactive SpRheb and not the

wild-type version was found to interact with Tor2 suggesting that the interaction between these two proteins is dependent upon GTP. This idea was further supported by the observation that wild-type SpRheb could only interact with Tor2 if it was expressed in the *tsc2* mutant cells and not in wild-type cells. Hyperactive mutants of mammalian Rheb have been identified [48]. In particular, S16H mutant exhibits increased GTP loading *in vivo* and is resistant to TSC1/TSC2 GAP *in vitro*. The mutant exhibits increased ability to promote the phosphorylation of S6K1 and 4E-BP1. In addition, RhebQ64L exhibits increased GTP loading [37]. Jiang and Vogt [49] showed that HsRheb mutants Q64L and N153T were hyperactive and had oncogenic potential. The oncogenic ability of Q64L was shown to depend upon the presence of an intact farnesylation motif (CaaX) as well as mTOR activity since no cellular transformation was observed if rapamycin was used.

Mutations in the effector domain have been reported. Mutations in the switch I region have significant effect on the function of Rheb. For example, D36A, P37A, T38A, and N41A mutants of human Rheb still retain GTP binding but are defective in mTORC1 activation *in vitro* [50]. In addition, N41A mutant of human Rheb was less effective in supporting the mTOR in the absence of nutrients [51]. Significance of the switch I region of fission yeast Rheb was shown by the lack of complementation of the *rheb* mutant cells by RhebE40K [27]. Other reports suggest that regions outside of switch I also play important roles for the ability of Rheb to activate mTOR signaling. An alanine-scanning mutagenesis study identified Y54A and L56A mutants of human Rheb as mutants defective in mTOR activation [51]. In addition, a systematic change of solvent exposed residues of human Rheb identified two mutants, Y67A/I69A and I76A/D77A, as mutants defective in the ability to stimulate mTOR signaling in the absence of nutrients [52]. These mutants still retain the ability to bind guanine nucleotides. Interestingly, the mutations are located in the switch II segment.

Using fission yeast Rheb, a new type of Rheb mutants has been obtained. Murai *et al.* [53] isolated two dominant active *rhb1* mutants, *rhb1-DA4* and *rhb1-DA8* of SpRheb. The *rhb1-DA4* carries a V17A mutation within the G1 box. This mutant is the same as the one that has been previously shown to act as a hyperactive mutant [47]. On the other hand, the *rhb1-DA8* mutant carries two mutations Q52R and I76F. While an amino acid uptake was prevented by both *rhb1-DA4* and *rhb1-DA8* mutants in a dominant fashion, only the *rhb1-DA4* mutant prevented the response to nitrogen starvation. This may suggest that these mutants act differently and can be used to genetically dissect the Rheb-dependent signaling cascade.

III. Activation of mTORC1 by Rheb

A. Initial Observations

Rheb functions as a critical player in the insulin/TOR/S6K signaling and is involved with the regulation of growth and cell cycle progression [12, 54]. Interaction of Rheb with mTOR has been reported [55, 56]. Use of Rheb mutants suggested that the interaction did not depend on the binding of GTP and in fact was stronger with a mutant that exists predominantly as a GDP-bound form [55, 56]. Effects of amino acid starvation on the interaction of Rheb and mTOR were reported [57]. Expression of different mTOR fragments in mammalian cells together with Rheb mapped the Rheb interaction site to the 2148–2300

residues of mTOR [55]. The interaction of Rheb and Tor2 in fission yeast was reported [47]. In this case, the use of hyperactivated form of Rheb was critical in the detection of the interaction, suggesting that the interaction is preferential with the GTP-bound form.

B. Rheb Activates mTORC1 *In Vitro*

More recently, Rheb was shown to activate mTOR *in vitro*, thus providing conclusive evidence that Rheb is an activator of mTOR [50, 58]. In these experiments, mTORC1 was isolated from cells starved for nutrients by using antibody against raptor. The complex is inactive, however, the addition of recombinant Rheb causes activation of mTORC1 as detected by the phosphorylation of substrate protein 4E-BP1 using antibody against phospho-4E-BP1 (Figure 3.2). The activation of mTORC1 by Rheb is dependent on the binding of GTP, as no activation was observed when GDP-bound Rheb or nucleotide free Rheb was used. The activation also was dependent on the presence of intact effector domain of Rheb. This point was shown by the use of Rheb mutants such as D36A and P37A.

The activation showed strong specificity for Rheb protein, as other members of the Ras family G-proteins such as RalA, RalB, KRas, RRas, Rad did not activate the mTORC1. Rho family G-proteins such as Rac1 and Cdc42 also did not activate mTORC1. In addition to Rheb, mammalian cells have another Rheb called Rheb2 (also called RhebL1). We have shown that Rheb2 also activates mTORC1 in the *in vitro* system [50]. Thus these two proteins have similar function but they differ in their tissue distribution [17].

It is important to point out that Rag G-proteins do not activate mTORC1 [30]. While Rag plays critical roles in the activation of mTORC1 in response to amino acid signaling, the effect appears to occur prior to the Rheb activation, as Rag cannot activate mTORC1 in the absence of Rheb [30].

Rheb activation is specific to mTORC1, as no dramatic effects of Rheb on mTORC2 were observed. In the experiment we carried out [50], mTORC2 was isolated from cells starved for nutrients (serum and amino acids) by using antibody against Rictor. Activity of the isolated complex was examined by using Akt as a substrate. No significant stimulation was observed by the addition of Rheb, suggesting that the effect of Rheb is preferentially on mTORC1.

The ability of Rheb to activate mTORC1 *in vitro* does not depend on the farnesylation of Rheb. In the *in vitro* systems, Rheb purified after expression in *E. coli* has been used and this unmodified form was sufficient to induce mTORC1 activation. When Rheb isolated from insect cells after baculovirus infection was used, no improvement of the efficacy was observed, suggesting that farnesylation does not provide improvement in potency of Rheb to activate mTORC1.

The fact that Rheb can activate mTORC1 *in vitro* suggests that Rheb directly interacts with mTORC1. This interaction may be transient, as it has been difficult to detect Rheb associated with mTORC1 after the incubation (unpublished). Perhaps the interaction is transient and strong interaction may be counterproductive for mTORC1 activation. This may

agree with the previous observations that the interaction between Rheb and mTOR is rather weak.

C. Rheb Enhances Recruitment of the Substrate Protein to mTORC1

Activation of mTORC1 occurs in two steps. In the first step, substrate protein is recruited to mTORC1 mediated by raptor that recognizes the TOS motif present in the substrate proteins such as 4E-BP1 or S6K. In the second step, kinase activity of mTOR phosphorylates the substrate protein. We have shown that the activation of Rheb induces enhanced recruitment of substrate proteins to mTORC1. This was shown by examining the association of 4E-BP1 with mTORC1 in the presence and absence of Rheb-GTP [50]. This assay was carried out in the absence of ATP to avoid turnover of the substrate protein. The Rheb-induced enhancement of 4EBP1 recruitment to mTORC1 was dependent on the presence of the intact effector domain of Rheb.

It is known that detergents used during the isolation of mTORC1 affect the association of raptor with mTOR [59]. While CHAPS can retain the interaction of Rheb and mTOR, NP40 disrupts the interaction. The mTORC1 isolated by using the buffer containing NP40 was not activated by Rheb, in agreement with the idea that raptor plays important roles in the Rheb-induced activation of mTORC1. PRAS40 inhibits mTORC1 activation by competing with substrate proteins, and the Rheb-induced activation of mTORC1 was inhibited by the addition of PRAS40. Intrinsic kinase activity of mTOR can be detected by autophosphorylation. The use of Mn^{2+} instead of Mg^{2+} in the kinase reaction promotes this reaction. We did not detect autophosphorylation of mTOR when Rheb was added. Thus, Rheb does not appear to induce intrinsic kinase activity.

These results obtained using the *in vitro* system are consistent with the idea that Rheb interacts with mTORC1 and enhances phosphorylation of substrate proteins (Figure 3.3). This involves increased recruitment of substrate proteins.

D. FKBP38 Appears not to Play a Major Role in the Activation of mTORC1 by Rheb

Bai *et al.* [60] have reported that Rheb binds FKBP38 in a GTP-dependent manner and that this relieves inhibitory effects of FKBP38 on mTORC1. The interaction between Rheb and FKBP38 was reported to be dependent on the presence of intact effector domain of Rheb [32]. However, the idea that Rheb activates mTORC1 indirectly by binding to FKBP38 is difficult to reconcile with the above observations with the *in vitro* system [50]. First, very little FKBP38 was detected in the mTORC1 preparation used for the *in vitro* reaction. Second, removal of FKBP38 from the cell extracts by the use of antibody against FKBP38 before preparing mTORC1 did not make differences in the ability of mTORC1 to be activated by Rheb. Finally, inhibition of FKBP38 expression by the use of siRNA only slightly affected the mTORC1 signaling. The mTORC1 isolated from these cells after nutrient starvation was still activated by Rheb. These results suggest that Rheb can activate mTORC1 in the absence of FKBP38. In addition, Uhlenbrock *et al.* failed to detect the interaction between Rheb and FKBP38 [61]. Conflicting results have been obtained regarding the effect of expression of FKBP38 on the mTOR signaling *in vivo* [44, 50, 61].

Taken together, it appears that FKBP38 is not a major player in the Rheb activation of mTORC1.

IV. Functions of Rheb that Are Independent of mTOR

The Ras superfamily G-proteins generally activates multiple downstream signaling pathways. For example, downstream effectors of Ras include Raf kinase, PI3 kinase, RalGDS, and Rin1 [62, 63]. Therefore, it is expected that multiple downstream effectors of Rheb will be discovered. A number of reports suggest that this is the case.

Involvement of the TSC/Rheb signaling on perinuclear aggresome formation has been reported [64]. The aggresome represents a mechanism to dispose of misfolded proteins that exceed the degradative capacity of proteasome and autophagy systems. TSC mutant cells are defective in aggresome formation and undergo apoptosis upon the accumulation of misfolded proteins. Involvement of Rheb in the aggresome formation is demonstrated using Rheb mutants, Rheb-Q64L and Rheb-D60K. Use of rapamycin suggests that these effects are independent of mTORC1.

Effects of Rheb on Raf kinase have been reported. Rheb was shown to antagonize NIH3T3 transformation by H-ras^{G12V} [19]. Rheb was found to bind Raf-1 *in vitro*. Inhibition of MAPK activation by the expression of Rheb was shown in *Xenopus* oocytes. Effects of Rheb on Raf were also reported by Yee and Worley [65]. This study showed that Rheb interacts with Raf-1 kinase and regulates cAMP and growth factor signaling in conjunction with H-Ras. More recently, the effect of Rheb on Raf kinases is reported by Karbowiczek *et al.* [66, 67]. Tuberin expression increases p42/44 MAP kinase phosphorylation and B-Ras activity. Expression of Rheb inhibits wild-type B-Raf but not activated forms of B-Raf. Inhibition of B-Raf and MAPK by Rheb is resistant to rapamycin, while Rheb activation of S6K is rapamycin sensitive.

Other suggested downstream effectors of Rheb include phospholipase D1 (PLD1) [68]. Significance of PLD1 in the activation of mTOR signaling was suggested from the use of 1-butanol, an inhibitor of PLase D. Furthermore, it is shown that phosphatidic acid, the product of PLase D, is required for the formation of mTORC1 and mTORC2 complexes [69]. In this scenario, Rheb affects PLase D leading to the regulation of mTORC1 formation.

V. Future Prospects

Studies over the years have established Rheb as a member of the Ras superfamily G-proteins. This G-protein has unique structural features and has a high GTP level. TSC1/TSC2 complex functions as a GAP for Rheb and this involves the use of the asparagine-thumb mechanism. Other regulators of Rheb need to be identified to understand the function of this G-protein in the regulation of a variety of cellular processes. A major area of study concerns identification of GEF for Rheb. While TCTP has been proposed to function as a GEF for Rheb, assignment of this protein as a Rheb GEF is premature at this point.

Involvement of Rheb in the mTORC1 signaling has been established by a number of experiments. Because Rheb can activate mTORC1 isolated from nutrient starved cells, Rheb

has to interact with mTORC1. However, this interaction may be transient and may not require strong stable binding. One of the consequences of the Rheb action is to enhance recruitment of substrate proteins to mTORC1, a process that is mediated by raptor. Further investigation of this *in vitro* system is needed to understand biochemical and structural consequences of the interaction of Rheb with mTORC1. Further studies on downstream effectors of Rheb may reveal multiple downstream pathways activated by Rheb. Use of Rheb mutants that may distinguish activation of different downstream signaling pathways may be valuable for this analysis.

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Fig. 3.1.
The Rheb family G-proteins. Structure of Rheb proteins from human, *Drosophila*, and fission yeast are shown. G1–G5 boxes are indicated and the sequence of the effector domain (G2) is shown. Identity and similarity among different Rheb proteins are shown.

Fig. 3.2.

Rheb G-protein specifically activates mTORC1 *in vitro*. (B) Recombinant Rheb but not other Ras superfamily G-proteins can activate mTORC1 isolated from HEK293T cells after nutrient starvation. 4E-BP1 was used as a substrate protein and its phosphorylation was detected on a SDS polyacrylamide gel after incubation with ATP. G-proteins tested are shown in (A). The reaction was dependent on GTP bound to Rheb (C). Both Rheb1 and Rheb2 activated mTORC1 *in vitro* (D). Reproduced from Ref. [50].

Fig. 3.3.

A model for the action of Rheb on mTORC1. Rheb in its GTP-bound state interacts with mTORC1 and activates it. This involves enhanced recruitment of substrate proteins resulting in their phosphorylation. Rheb-GTP is converted to Rheb-GDP by the action of TSC1/TSC2 GAP.