J. Biochem. **137**, 423–430 (2005)
DOI: 10.1093/jb/mvi046

Novel Role of the Small GTPase Rheb: Its Implication in Endocytic Pathway Independent of the Activation of Mammalian Target of Rapamycin

Kota Saito, Yasuhiro Araki, Kenji Kontani, Hiroshi Nishina and Toshiaki Katada*

Department of Physiological Chemistry, Graduate School of Pharmaceutical Sciences, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033

Received January 8, 2005; accepted February 1, 2005

The Ras-homologous GTPase Rheb that is conserved from yeast to human appears to be involved not only in cell growth but also in nutrient uptake. Recent biochemical analysis revealed that tuberous sclerosis complex (TSC), a GTPase-activating protein (GAP), deactivates Rheb and that phosphatidylinositol 3′-kinase (PI3k)-Akt/PKB kinase pathway activates Rheb through inhibition of the GAP-mediated deactivation. Although mammalian target of rapamycin (mTOR) kinase is implicated in the downstream target of Rheb, the direct effector(s) and exact functions of Rheb have not been fully elucidated. Here we identified that Rheb expression in cultured cells induces the formation of large cytoplasmic vacuoles, which are characterized as late endocytic (late endosome- and lysosome-like) components. The vacuole formation required the GTP form of Rheb, but not the activation of the downstream mTOR kinase. These results suggest that Rheb regulates endocytic trafficking pathway independent of the previously identified mTOR pathway. The physiological roles of the two Rheb-dependent signaling pathways are discussed in terms of nutrient uptake and cell growth or cell cycle progression.

Key words: late endosome, lysosome, mTOR, Rheb, small GTPase.

Abbreviations: DMEM, Dulbecco's modified Eagle's minimum essential medium; GAP, GTPase-activating protein; mTOR, mammalian target of rapamycin; PI3k, phosphatidylinositol 3′-kinase; Rheb, *ras* homologue enriched in brain; TSC, tuberous sclerosis complex.

Members of the small GTPase Ras superfamily act as molecular switches that regulate a wide range of cellular functions including cell proliferation and differentiation. Ras homologue enriched in brain, Rheb, is a member of an atypical family of Ras-related G proteins, of which sequences in the GTP-binding domain have not been conserved (*1*). Di-Ras1, Di-Ras2, and ARHI also belong to the atypical GTPase family (*2*). Rheb was initially identified as a gene whose mRNA expression is increased in rat brain by seizures or by the stimulation of long-term potentiation (*3*). Recently, Rheb has received significant attention, since it was identified as a positive regulator of the mammalian target of rapamycin (mTOR) pathway, which regulates cell growth in response to various growth factors, cellular energy and nutrient levels (*4–10*). Moreover, the TSC1/TSC2 complex, of which mutations leads to tuberous sclerosis complex (TSC), was found to be a GTPase-activating protein (GAP) for Rheb (*5–8*, *11*).

From recent biochemical studies in mammalian cells, Rheb-related signaling pathways leading to cell growth may be summarized as follows. The stimulation of growth factor receptors at the cell surface results in the activation of phosphatidylinositol 3′-kinase (PI3k), which then phosphorylates Akt/PKB (*12*, *13*). The activated Akt phosphorylates TSC2 to negatively regulate its GAP activity toward Rheb (*6*, *14*, *15*). This results in the accumulation of GTP-Rheb and stimulates the mTOR kinase activity (*4–10*). Phosphorylation of p70 ribosomal S6 kinase (S6K) and the translation initiation factor 4E-BP1, which are responsible for the increase in protein synthesis, has been implicated in the downstream events of mTOR kinase (*16*, *17*). Thus, Rheb is involved in the signaling pathways responsible for cell growth and cell cycle progression.

On the other hand, Rheb appears to play an important role in intracellular uptake of nutrients such as amino acids. Rheb is conserved from yeast to human, and recent genetic studies have contributed significantly to the understanding of its function. In yeast, arginine uptake was regulated positively and negatively by TSC and Rheb, respectively (*18–20*). In addition, Tsc1 and Tsc2 knockouts in *Schizosaccaromyces pombe* exhibited abnormal intracellular distribution of an amino-acid permease (*21*). In human, TSC patients suffer from seizures and epilepsy, which may be related to the dysfunction of extracellular glutamate uptake in glial cells. The accumulated glutamate in the synaptic cleft may cause neuronal cell death. However, the linkage between Rheb activation and nutrient uptake and the molecular mechanism underlying TSC epileptogenesis have not been fully clarified.

In the present study, we found that Rheb activation induces the formation of large vacuoles in the cytoplasm

*To whom correspondence may be addressed. Tel: +81-3-5841-4750, Fax: +81-3-5841-4751, E-mail: katada@mol.f.u-tokyo.ac.jp

of Rheb-transfected cells, and that the vacuoles are characterized as late endosome- or lysosome-like organelles. The large vacuole formation was dependent on the GTPase cycle of Rheb and suppressed by inhibition of the PI3k activity. However, inhibition of mTOR kinase did not affect the Rheb-induced vacuole formation. These results suggest that Rheb plays an important role in endocytic trafficking pathway independent of the previously identified mTOR pathway that leads to cell growth and cell-cycle progression. Possible roles of Rheb are discussed in terms of the relationship between the Rheb-dependent endocytic pathway and nutrient uptake.

MATERIALS AND METHODS

Northern Blot Analysis—Expression patterns of Rheb and Rheb2 mRNAs were analyzed using human multiple tissue membranes (BD Biosciences). Full-length sequences of Rheb and Rheb2 that had been radiolabeled with [α-^{32}P]dCTP were hybridized to the membranes overnight at 65°C in the Expresshyb solution (BD Biosciences), and the membranes were subjected to autoradiography (*22*).

Cell Culture and Transfection—MDCK, HeLa, 1321N1, and 293 cells were maintained in Dulbecco's modified Eagle's minimum essential medium (DMEM) supplemented with 10% fetal bovine serum. The cells cultured in a 35-mm dish were transfected with 1 μg of DNA and 2 μl of LipofectAMINE 2000 (Invitrogen) in Opti-MEM according to the manufacturer's protocol and incubated at 37°C for 16 h unless otherwise specified.

Confocal Microscopic Analysis—The transfected cells in a glass-based dish (35-mm diameter, Matsunami) were analyzed by Carl Zeiss confocal microscopy with LSM510 (*23*). For the analysis of dextran uptake and the labeling of acidic organelles (see Fig. 4), the transfected MDCK cells were further incubated at 37°C with 1 mg/ml of FITC-dextran (Molecular Probes) for 12 h (Fig. 4A) and 50 nM Lysotracker Green (Molecular Probes) for 1 h (Fig. 4B), then washed for four times with PBS before the confocal microscopic analysis. In some experiments (see Fig. 4C), MDCK cells were first incubated with 1 mg/ml of FITC-dextran in DMEM at 37°C for 4 h and further cultured without the dextran for an additional 20 h. The dextran-preloaded cells were transfected with Dsred-Rheb and incubated for the indicated times before the confocal microscopic analysis.

Analysis of Akt Kinase Activity—293 cells cultured in a 6-cm dish were transfected with 1.5 μg of Myc-Akt and 1.5 μg of FLAG or FLAG-Rheb and further incubated in the presence or absence of 50 μM LY294002 (Calbiochem) for 16 h. The cells were solubilized with an extraction buffer consisting of 40 mM Na-Hepes (pH 7.4), 75 mM NaCl, 1% Triton X-100, 1 mM EDTA, 15 mM NaF, 1 mM Na_3VO_4, 10 mM $Na_4P_2O_7$, 1 μg/ml leupeptin, and 2 μg/ml aprotinin, and the cell lysate was immunoprecipitated with Myc-antibody (Sigma) conjugated to Protein G-agarose (Amersham Biosciences). The immunocomplexes were washed three times with a buffer consisting of 50 mM Na-Hepes, 100 mM NaCl, 0.2% Triton X-100, 15 mM NaF, 1 mM Na_3VO_4, 10 mM $Na_4P_2O_7$, 4 mM EDTA, 1 mM DTT, and 2 μg/ml aprotinin, then twice with a kinase buffer consisting of 50 mM Na-Hepes, 0.01% Triton X-100, 1 mM DTT, 10 mM $MgCl_2$. They were then incubated with the kinase buffer further supplemented with 50 μM ATP, 3 μCi [γ-^{32}P]ATP, and 10 μg/ml of GST-GSK3β at 37°C for 20 min. Phosphorylated proteins were analyzed by SDS-PAGE, and the radioactivity was visualized with a BAS-1800 bioimaging analyzer (Fuji Firm).

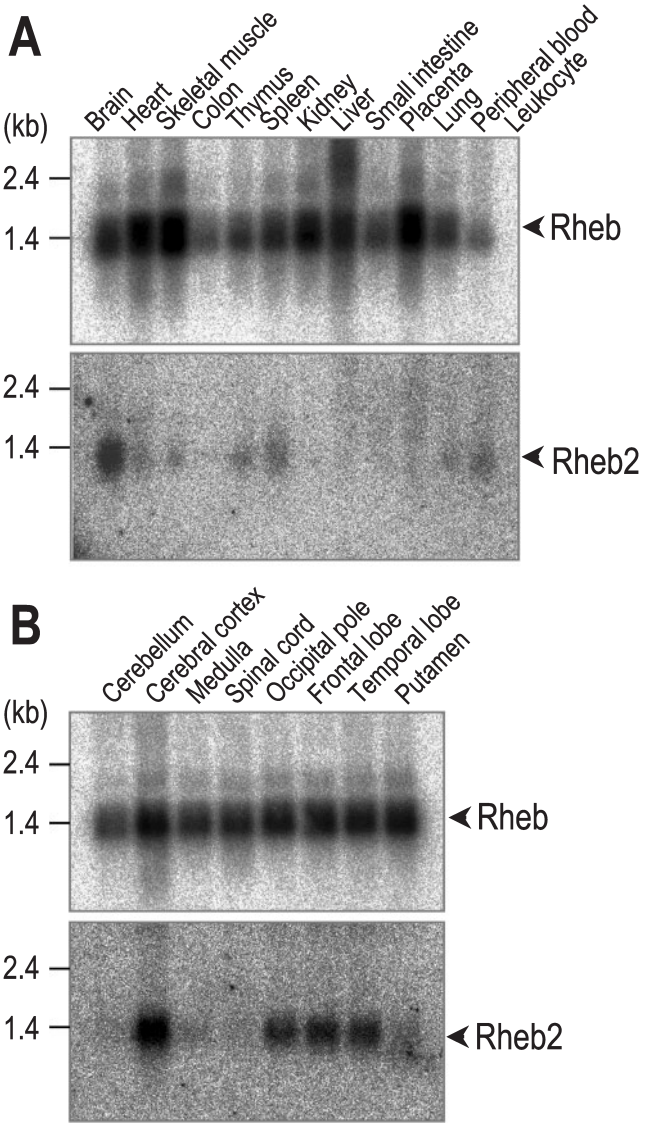

Fig. 1. **Northern blot analysis of Rheb and Rheb2.** Membranes blotted with RNAs obtained from various human tissues (A) and the brain regions (B) were hybridized with ^{32}P-labeled Rheb (top panels) and Rheb2 (bottom panels) probes, respectively, as described under "MATERIALS AND METHODS."

Analysis of mTOR Kinase Activity—293 cells in a 6-cm dish were transfected with 1.5 μg of HA-S6K and 1.5 μg of FLAG or FLAG-Rheb and further incubated in the presence or absence of 40 nM rapamycin (Calbiochem) for 5 h. The cells were washed with PBS and further cultured in DMEM supplemented with 0.1% BSA in the presence or absence of rapamycin for additional 12 h. The cells were solubilized with the extraction buffer, and the cell lysate was immunoprecipitated with anti-HA affinity

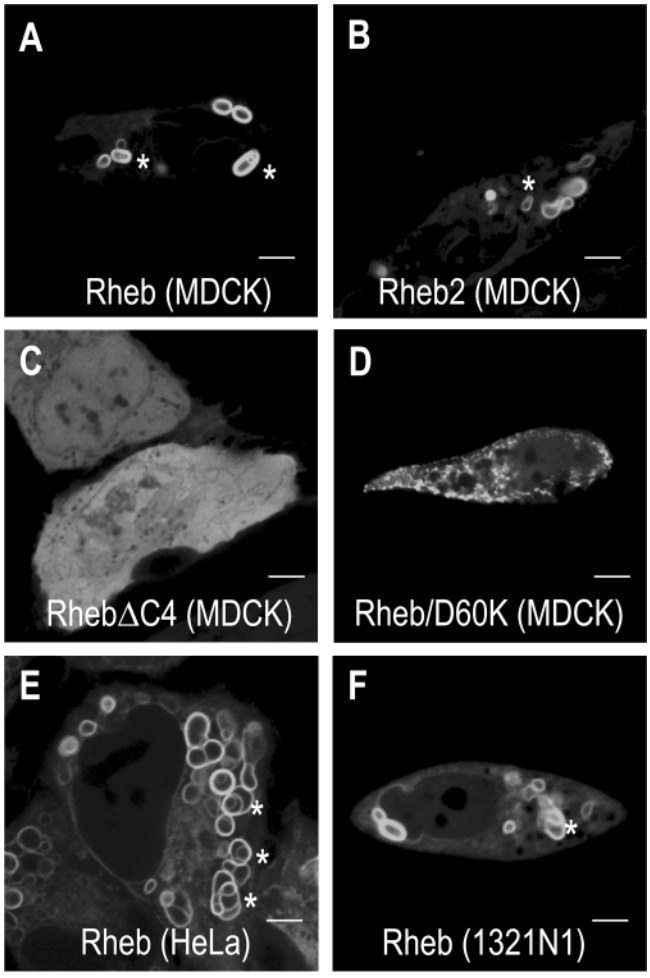

Fig. 2. **Rheb and Rheb2 induce large vacuole formation.** MDCK cells were transfected with EGFP-Rheb (A), EGFP-Rheb2 (B), EGFP-RhebΔC4 (C), or EGFP-Rheb/D60K (D) and cultured for 16 h. HeLa (E) and 1321N1 astrocytoma (F) cells were also transfected with EGFP-Rheb. These cells were analyzed by a confocal microscopy as described under Experimental Procedures. Scale bars indicate 5 μm, and asterisks show the multi-lamellar and multi-vesicular structures.

matrix (Roche). The immunocomplexes were washed five times with a buffer consisting of 20 mM Tris-HCl (pH 7.5), 2 mM EDTA, 100 mM NaCl, and 0.4% Triton X-100, and the precipitated proteins were subjected to SDS-PAGE and Western blotting with anti-p70 (Santa Cruz Biotechnology) and anti-phosphorylated p70 (Cell Signaling) S6K antibodies.

All experiments were repeated at least three times with different batches of the cell samples, and the results were fully reproducible. Hence, most of the data shown are representative of several independent experiments.

RESULTS AND DISCUSSION

Rheb and Rheb2 mRNAs Are Differently Distributed in Human Tissues—In addition to Rheb (Rheb1), human Rheb2 cDNA was recently identified (*4*). To investigate the distribution patterns of Rheb and Rheb2 mRNAs in various human tissues, Northern blot analysis was performed with a radiolabeled probe containing full-length sequences of Rheb and Rheb2. As shown in Fig. 1A, a 1.6-kb Rheb transcript was observed in various human tissues in accordance with a previous report (*3*). In contrast, a 1.6-kb Rheb2 transcript was observed predominantly in the brain and less in the spleen and peripheral blood. Because of the highly sequence homology between Rheb and Rheb2 mRNAs, the specificity of each probe was confirmed by the following analysis. Nitrocellulose membranes were blotted with Rheb and Rheb2 plasmids and hybridized with both probes. Specific signals were observed only when their RNAs were hybridized with the corresponding probes (data not shown). Thus, no cross-hybridization between Rheb and Rheb2 was detected under the present conditions. We next examined the distributions of Rheb and Rheb2 mRNAs in specific brain regions. Rheb was found to be expressed in all brain regions, while Rheb2 mRNA was restricted to the cerebral cortex, occipital pole, frontal and temporal lobes (Fig. 1B). Rheb2 is present only in mammalian cells, although Rheb is highly conserved among various species. Thus, Rheb2 may have a specific role in the brain region of higher eukaryotes, of which activation might lead to epilepsy, which is a typical pathology of TSC patients.

Rheb Induces Large Vacuole Formation—To determine the intracellular localization of Rheb and Rheb2 in living cells, EGFP-tagged proteins were expressed in MDCK cells, and their distributions were analyzed by confocal microscopy (Fig. 2, A–D). Interestingly, the expression of Rheb and Rheb2 induced the formation of large vacuoles dispersed throughout the cells, and the proteins localized to the vacuole membranes (see panels A and B). In contrast, the expression of a mutant Rheb, RhebΔC4, which lacks the carboxy-terminal membrane-anchoring sequence CAAX, give rise to diffused signals and did not induce such large vacuoles (Fig. 2C). Enlarged vacuoles were also induced upon the expression of EGFP-tagged Rheb in HeLa, 1321N1 human astrocytoma cells (Fig. 2, E and F) and 293 cells (see Figs. 5B and 6B, control cells). As shown by the asterisks in Fig. 2, these vacuoles often had multi-lamellar structures, which are characteristic of late endocytic vesicles (late endosome and lysosome).

Rheb Localizes to Endocytic Rab7- and Rab9-Positive Vesicles—To identify the nature of the large vacuoles in which Rheb localized, some members of the Rab GTPase family tagged with Dsred were simultaneously expressed in MDCK cells as organelle markers specific for each subcellular compartment. As shown in Fig. 3, A and D, Rab5 and Rab11, which are involved in early endosome formation and endosomal recycling, respectively, showed distinct staining patterns from Rheb. On the other hand, Rab7 and Rab9, used as late endocytic markers, mostly co-localized with Rheb in the enlarged vacuoles (Fig. 3, B and C). These data correlate with the observation that Rheb localizes in the multi-lamellar late endocytic vesicles. When the amount of Rheb transfected was increased, we observed partial co-localization of Rab5 in the Rheb-induced vacuoles (data not shown), suggesting that hybrids of early and late endocytic organelles were created.

Vacuole Formation Is Affected by the GTPase Cycle of Rheb—Rheb has been characterized as a unique small GTPase in which critical amino acids necessary for

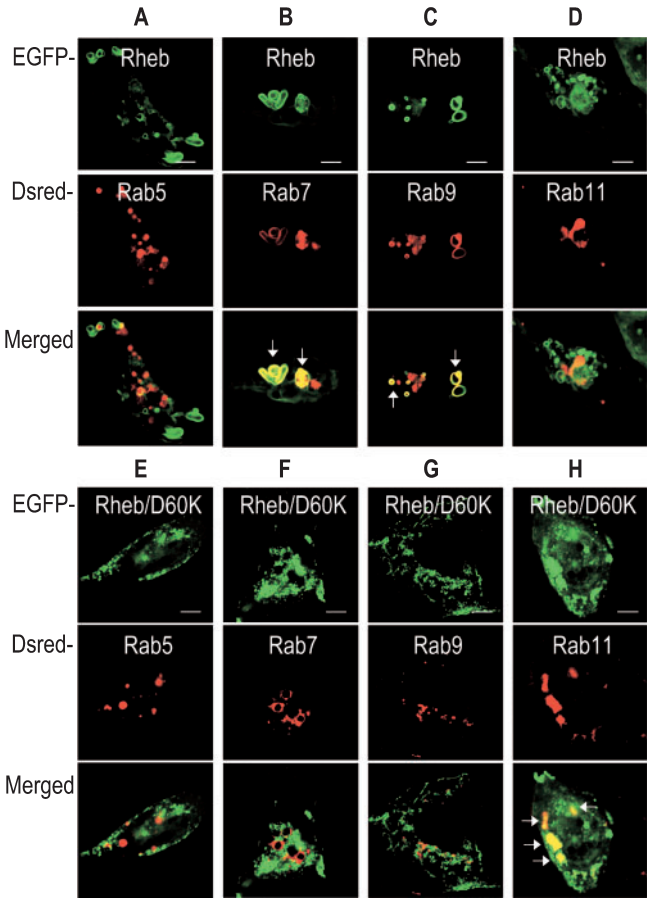

Fig. 3. **Rheb co-localizes with Rab7 and Rab9 in multi-vesicular structures, whereas Rheb/D60K mutant co-localizes partly with Rab11-containing vesicles.** MDCK cells were transfected with EGFP-Rheb (A–D) or EGFP-Rheb/D60K (E–H), together with Dsred-Rab5 (A and E), Dsred-Rab7 (B and F), Dsred-Rab9 (C and G) or Dsred-Rab11 (D and H), and further incubated for 16 h. The fluorescence of EGFP (upper) and Dsred (middle) was visualized by the confocal microscopy. Merged images of the two signals are also illustrated (bottom). Scale bars indicate 5 μm.

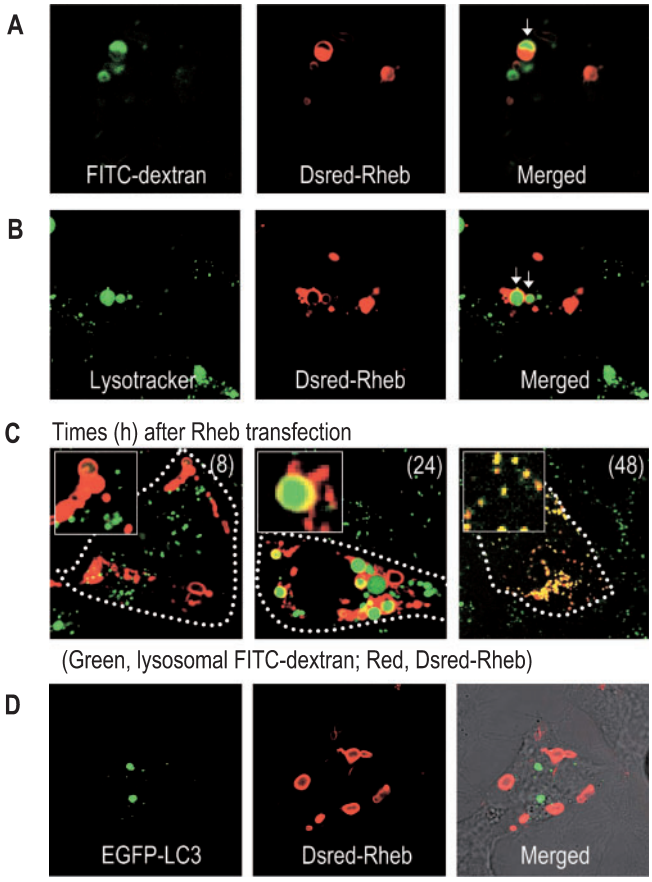

Fig. 4. **Rheb-positive vesicles are translocated from late endosomes to lysosomes.** A and B, MDCK cells transfected with Dsred-Rheb (A and B) were incubated with 1 mg/ml of FITC-dextran for 12 h (A) or 50 nM Lysotracker Green for 1 h (B). C, MDCK cells, of which lysosomal fractions had been labeled with FITC-dextran, were transfected with Dsred-Rheb and further incubated for the indicated times. The left upper insets show the enlargement of a part of the images, and the white dotted line indicates a single Rheb-transfected cell. D, MDCK cells were transfected with EGFP-LC3 and Dsred-Rheb and cultured for 16 h. The fluorescent and merged images are illustrated.

GTPase reaction are not conserved (*3*). This implies that Rheb may exist in living cells mostly as a GTP-bound form (*1*). Furthermore, a mutant Rheb, Rheb/D60K, has recently been reported to exhibit a dominant-negative effect on the activation of the mTOR pathway (*24*). To confirm the nucleotide-bound status of the wild-type and mutant Rhebs, HeLa cells were transfected with the FLAG-tagged proteins and cultured in $^{32}P_i$-containing medium. The recombinant proteins were immunoprecipitated, and the radiolabeled nucleotides associating with the proteins were analyzed by thin-layer chromatography. As expected, wild-type Rheb and Rheb/D60K existed mostly as GTP-bound and GDP-bound forms, respectively (data not shown).

We next examined how the nucleotide-bound forms of Rheb are localized in MDCK cells. In contrast to wild-type Rheb (see Fig. 2A), the expression of Rheb/D60K did not induce large vacuoles, but the mutant protein localized in intracellular small vesicles (Fig. 2D). As shown in Fig. 3, F and G, co-staining with Rab7 and Rab9 revealed that Rheb/D60K localized was quite differently from the wild-type protein, but abutting on late endocytic vesicles (compare with B and C). Rab5 also did not co-localize with the Rheb/D60K signals (E). However, recycling endosomes associating with Rab11 showed partial co-localization with Rheb/D60K (H). It should be noted here that the vesicular localization of each Rab protein was not markedly affected by the transfection of the wild-type or the mutant Rheb proteins (compare panels A–D with E–H). These data suggest that the subcellular localization of Rheb changed depending on its nucleotide-bound state.

Rheb-Positive Vesicles Are Translocated from Late Endosomes to Lysosomes—We further investigated how the Rheb-positive vesicles are created in the membrane trafficking pathway by comparing the localization of various endocytic makers. As shown in Fig. 4A, FITC-dextran, which is a non-selective endocytic marker that is able to reach late endosomes and lysosomes, was found to be present partly in lumens of the Rheb-positive vesicles. Lysotracker, a labeling probe for acidic organelles such as lysosomes (*25*), also accumulated in the Rheb-positive structures (Fig. 4B), indicating that parts of the Rheb-positive vesicles had acidic characteristics. We also investigated the time-dependent formation of Rheb-positive vesicles in MDCK cells, of which the lysosomal fraction had been selectively labeled with FITC-dextran. As shown in Fig. 4C, Rheb-positive vesicles were evident at 8 h after the transfection, but their distribution never co-localized with FITC-dextran, indicating that the early stage of expressed Rheb is not present in lysosomes. However, some Rheb signals co-localized with the FITC-dextran at 24 h, and most of the Rheb signals moved to the FITC-labeled lysosome at 48 h, suggesting that Rheb vesicles are gradually translocated to lysosomal compartments. These results are consistent with the previous results showing the co-localization of Rheb with Rab7 and Rab9 (see Fig. 3, B and C). Thus, the Rheb-positive vesicles appeared to be created at least from endocytic (or unidentified) organelles and gradually to fuse with lysosomes through late endosomes.

To further confirm the late endocytic localization of Rheb signals, we attempted to immunostain the Rheb-transfected cells with antibodies specific for late endocytic organelles. However, the Rheb-containing vesicles were fragmented during the process of fixation, and the localization of Rheb in the fixed cells appeared to be different from that in the living cells (data not shown). We could not overcome this problem with any of several fixation methods tested. Nevertheless, we would like to propose that the Rheb-induced vesicles have characteristics of late endocytic organelles based on the following observations. First, Rheb could co-localize with Rab7 and Rab9 but not with Rab5 or Rab11. Second, Rheb-induced organelles contained the endocytic marker FITC-dextran. Third, Rheb-induced vesicles were characterized as acidic organelles. Fourth, Rheb-containing vesicles gradually became positive for lysosomal markers.

The Rheb-positive vesicles seemed to share similar characteristics with autophagosomes in terms of Rab7-positive, lysosome-fusible, and multi-lamellar structures (*26*, *27*). Moreover, mTOR, which is a downstream kinase of Rheb GTPase, appears to function as a negative regulator of autophagy (*17*, *28*). Therefore, we analyzed whether Rheb signals co-localize with the autophagosome marker LC3, since Rheb-positive structures might be parts of accumulated incomplete autophagosomes due to the inhibition of autophagy through the activation of mTOR kinase. However, EGFP-LC3 signals never co-localized with Dsred-Rheb (Fig. 4D). When the transfected cells were starved by serum depletion, there was a marked elevation of the LC3 signals lacking co-localization with Rheb (data not shown). These data suggest that Rheb-positive vesicles are different entities from autophagosomes.

PI3k-Akt Signaling May Be Involved in the Upstream Pathway of the Rheb-Induced Vacuole Formation—As mentioned before, PI3k-Akt signaling appears to be involved in the upstream pathway of Rheb activation. To investigate whether inhibition of PI3k affects the Rheb-induced vacuole formation, the transfected 293 cells were incubated with the specific inhibitor LY294002, under which conditions PI3k activity was entirely suppressed. The effectiveness of the sustained PI3k inhibition was confirmed by measuring the phosphorylation of GSK-3β, a direct substrate of Akt. As shown in Fig. 5A, the inhibition of PI3k was clearly evident even after incubation of the cells with LY294002 for 16 h. Under these conditions, Rheb-induced large vacuole formation was markedly impaired in 293 cells (Fig. 5B). Instead, small punctated Rheb-positive vesicles were evident in the LY294002-treated cells, which are reminiscent of Rheb/D60K-induced vesicles (see Fig. 2D). This suggests that LY294002 probably activates endogenous GAP, so that expressed Rheb is rapidly deactivated and fails to induce large vacuoles under these conditions. These results may indicate that PI3k-Akt signaling is situated in the upstream of the pathway involved in the large vacuole formation. However, the PI3k activity has been also known to regulate various aspects of endosomal and lysosomal functions and membrane trafficking independent of Rheb functions (*29*). Thus, the GAP-sensitive GTPase cycle of Rheb appears to partly regulate the endocytic pathway from late endosomes to lysosomes, although we can not totally rule out the possibility that impaired vacuole formation in LY294002-treated cells is due to the inhibition of other

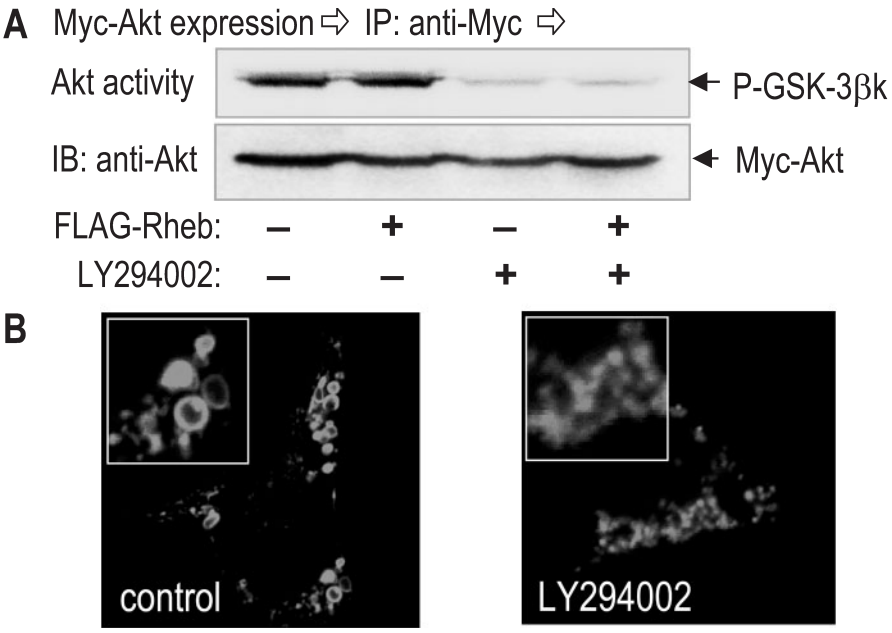

Fig. 5. **PI3k-Akt signaling may be involved in the Rheb-induced vacuole formation.** 293 cells that had been transfected with Myc-Akt and FLAG-Rheb (A) or Dsred-Rheb (B) were incubated in the presence or absence of 50 μM LY294002. A, the cell lysates were prepared and subjected to immunoprecipitation (IP) with an anti-Myc antibody, and the kinase activity and protein amount of Akt in the precipitated fractions were measured as described under "MATERIALS AND METHODS." B, the fluorescence of Dsred was visualized by confocal microscopy. The left upper insets show the enlargement of a part of the images.

endosomal target(s), which is regulated by the activity of PI3k.

Rheb-Induced Vacuolar Formation Is Independent of the Activation of mTOR—It has been reported that Rheb stimulates the kinase activity of mTOR, which leads to the activation of S6K responsible for protein synthesis (*16*). To investigate whether the activation of mTOR kinase is necessary for the induction of Rheb-positive vacuoles, 293 cells were transfected with FLAG-Rheb under conditions whereby mTOR activity was entirely suppressed with the specific inhibitor rapamycin. The effectiveness of the mTOR inhibition was confirmed by measuring the phosphorylation of p70 S6K, a direct substrate of mTOR. As shown in Fig. 6A, the inhibition of mTOR was clearly observed even after prolonged incubation of the cells with rapamycin. However, there was no marked difference in the Rheb-positive vacuole formation between the control and rapamycin-treated cells (Fig. 6B). These data indicate that the activation of mTOR is not required for the Rheb-induced vacuole formation.

The Possible Role of Rheb-Induced Vacuole Formation in Cell Functions Other than Cell Growth and Cell-Cycle Progression—The present study has revealed that Rheb activation induces large vacuoles in cytoplasm of the

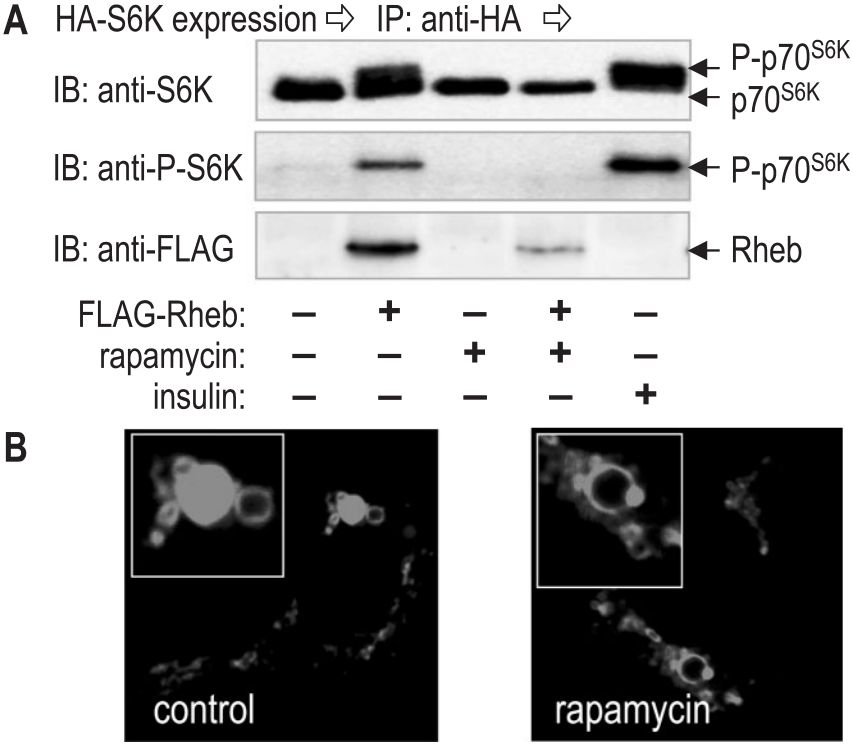

Fig. 6. **Activation of mTOR kinase is not required for the Rheb-induced vacuole formation.** 293 cells that had been transfected with FLAG-Rheb and HA-p70 S6K (A) or Dsred-Rheb (B) were incubated in the presence or absence of 40 nM rapamycin. A, as a control, the transfected HA-p70 S6 kinase was stimulated by incubation of cells with 200 nM insulin. Cell lysates were prepared and immunoprecipitated with an anti-HA antibody. The precipitated fractions were separated by SDS-PAGE and subjected to immunoblot analysis with anti–p70 S6K (upper) or anti–phosphorylated p70 S6K (middle) antibodies. The cell lysates were also immunoblotted with an anti-FLAG antibody (bottom). B, the fluorescence of Dsred was visualized by confocal microscopy. The left upper insets show the enlargement of a part of the images.

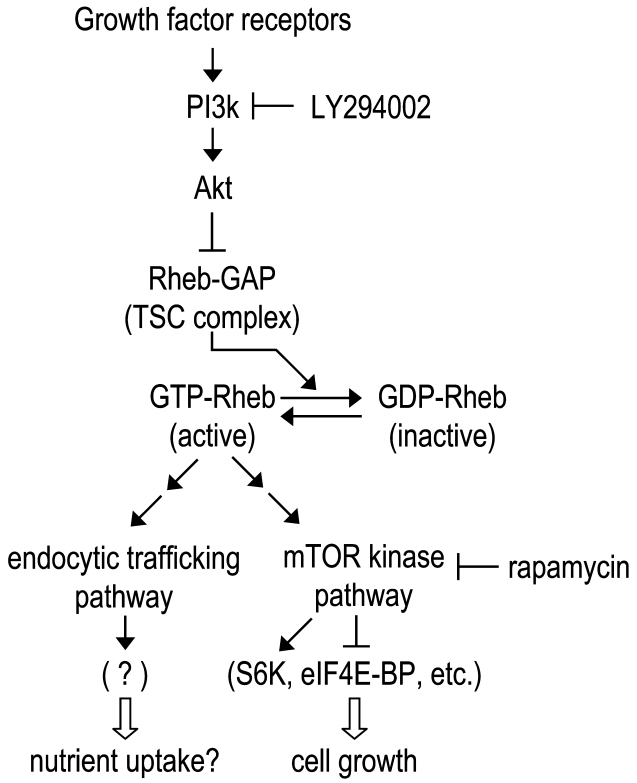

Fig. 7. **Schematic representation of Rheb-related signal transduction pathways.** Rheb induces not only the activation of mTOR kinase leading to cell growth but also late-endocytic vesicle formation that may play a critical role in nutrient uptake. See text for further explanation.

transfected cells and that the vacuoles are characterized as late endosome- or lysosome-like organelles. Moreover, the large vacuole formation is dependent on the GTPase cycle of Rheb and probably the stimulation of the PI3k-Akt pathway, but not on the activation of mTOR kinase. These data suggest that Rheb-induced vacuole formation is a novel Rheb-downstream pathway that is distinguishable from the activation of mTOR kinase pathway (Fig. 7). Rheb was first identified as a gene whose mRNA expression is increased in rat brain by seizures or by the stimulation of long-term potentiation (*3*). Since then, several groups has examined whether Rheb is involved in the activation of Raf, the well-known downstream kinase of conventional Ras (*30–33*). The recent identification of Rheb as a positive regulator of mTOR pathway has attracted considerable attention, in addition to the report that TSC acts as a GAP for Rheb (*1*, *15*, *34*, *35*). To our knowledge, however, this is the first report that indicates the involvement of Rheb in the endocytic pathway.

In yeast, several reports suggest that TSC positively and Rheb negatively regulate arginine uptake (*18–20*). In addition, Tsc1 and Tsc2 knockouts in *S. pombe* exhibited abnormal intracellular distribution of an amino-acid permease (*21*). It is, therefore, tempting to speculate that the activation of Rheb induces endocytic vacuoles that may have an effect on the localization of permeases. Our preliminary experiments indicate that Rheb is capable of interacting directly with several channels or transporters implicated in nutrient uptake. Moreover, these transporters were selectively translocated from the plasma membrane to Rheb-induced vacuoles in Rheb-transfected cells. These results suggest that Rheb-induced vacuoles are necessary for nutrient regulation through their translocation (Fig. 7). Further investigation is needed to uncover the physiological roles of Rheb, and it is important to investigate the regulation of the endocytic pathway in terms of nutrient uptake, in which Rheb-pathway may play a critical role.

This work was supported in part by research grants from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of the Japanese Government, the Japan Society for the Promotion of Science (JSPS), the Mitsubishi Foundation, and the Uehara Memorial Foundation.

REFERENCES

1. Li, Y., Corradetti, M.N., Inoki, K., and Guan, K.L. (2004) TSC2: filling the GAP in the mTOR signaling pathway. *Trends Biochem. Sci.* **29**, 32–38
2. Kontani, K., Tada, M., Ogawa, T., Okai, T., Saito, K., Araki, Y., and Katada, T. (2002) Di-Ras, a distinct subgroup of ras family GTPases with unique biochemical properties. *J. Biol. Chem.* **277**, 41070–41078
3. Yamagata, K., Sanders, L.K., Kaufmann, W.E., Yee, W., Barnes, C.A., Nathans, D., and Worley, P.F. (1994) rheb, a growth factor- and synaptic activity-regulated gene, encodes a novel Ras-related protein. *J. Biol. Chem.* **269**, 16333–16339
4. Patel, P.H., Thapar, N., Guo, L., Martinez, M., Maris, J., Gau, C.L., Lengyel, J.A., and Tamanoi, F. (2003) *Drosophila* Rheb GTPase is required for cell cycle progression and cell growth. *J. Cell Sci.* **116**, 3601–3610
5. Inoki, K., Li, Y., Xu, T., and Guan, K.L. (2003) Rheb GTPase is a direct target of TSC2 GAP activity and regulates mTOR signaling. *Gene. Dev.* **17**, 1829–1834
6. Garami, A., Zwartkruis, F.J., Nobukuni, T., Joaquin, M., Roccio, M., Stocker, H., Kozma, S.C., Hafen, E., Bos, J.L., and Thomas, G. (2003) Insulin activation of Rheb, a mediator of mTOR/S6K/4E-BP signaling, is inhibited by TSC1 and 2. *Mol. Cell* **11**, 1457–1466
7. Castro, A.F., Rebhun, J.F., Clark, G.J., and Quilliam, L.A. (2003) Rheb binds tuberous sclerosis complex 2 (TSC2) and promotes S6 kinase activation in a rapamycin- and farnesylation-dependent manner. *J. Biol. Chem.* **278**, 32493–32496
8. Tee, A.R., Manning, B.D., Roux, P.P., Cantley, L.C., and Blenis, J. (2003) Tuberous sclerosis complex gene products, Tuberin and Hamartin, control mTOR signaling by acting as a GTPase-activating protein complex toward Rheb. *Curr. Biol.* **13**, 1259–1268
9. Saucedo, L.J., Gao, X., Chiarelli, D.A., Li, L., Pan, D., and Edgar, B.A. (2003) Rheb promotes cell growth as a component of the insulin/TOR signalling network. *Nat. Cell Biol.* **5**, 566–571
10. Stocker, H., Radimerski, T., Schindelholz, B., Wittwer, F., Belawat, P., Daram, P., Breuer, S., Thomas, G., and Hafen, E. (2003) Rheb is an essential regulator of S6K in controlling cell growth in *Drosophila*. *Nat. Cell Biol.* **5**, 559–565
11. Zhang, Y., Gao, X., Saucedo, L.J., Ru, B., Edgar, B.A., and Pan, D. (2003) Rheb is a direct target of the tuberous sclerosis tumour suppressor proteins. *Nat. Cell Biol.* **5**, 578–581
12. Franke, T.F., Yang, S.I., Chan, T.O., Datta, K., Kazlauskas, A., Morrison, D.K., Kaplan, D.R., and Tsichlis, P.N. (1995) The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase. *Cell* **81**, 727–736
13. Burgering, B.M. and Coffer, P.J. (1995) Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction. *Nature* **376**, 599–602

14. Inoki, K., Li, Y., Zhu, T., Wu, J., and Guan, K.L. (2002) TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. *Nat. Cell Biol.* **4**, 648–657
15. Manning, B.D. and Cantley, L.C. (2003) Rheb fills a GAP between TSC and TOR. *Trends Biochem. Sci.* **28**, 573–576
16. Fingar, D.C. and Blenis, J. (2004) Target of rapamycin (TOR): an integrator of nutrient and growth factor signals and coordinator of cell growth and cell cycle progression. *Oncogene* **23**, 3151–3171
17. Raught, B., Gingras, A.C., and Sonenberg, N. (2001) The target of rapamycin (TOR) proteins. *Proc. Natl Acad. Sci. USA* **98**, 7037–7044
18. van Slegtenhorst, M., Carr, E., Stoyanova, R., Kruger, W.D., and Henske, E.P. (2004) Tsc1+ and tsc2+ regulate arginine uptake and metabolism in *Schizosaccharomyces pombe*. *J. Biol. Chem.* **279**, 12706–12713
19. Urano, J., Tabancay, A.P., Yang, W., and Tamanoi, F. (2000) The *Saccharomyces cerevisiae* Rheb G-protein is involved in regulating canavanine resistance and arginine uptake. *J. Biol. Chem.* **275**, 11198–11206
20. Mach, K.E., Furge, K.A., and Albright, C.F. (2000) Loss of Rhb1, a Rheb-related GTPase in fission yeast, causes growth arrest with a terminal phenotype similar to that caused by nitrogen starvation. *Genetics* **155**, 611–622
21. Matsumoto, S., Bandyopadhyay, A., Kwiatkowski, D.J., Maitra, U., and Matsumoto, T. (2002) Role of the Tsc1-Tsc2 complex in signaling and transport across the cell membrane in the fission yeast *Schizosaccharomyces pombe*. *Genetics* **161**, 1053–1063
22. Saito, K., Murai, J., Kajiho, H., Kontani, K., Kurosu, H., and Katada, T. (2002) A novel binding protein composed of homophilic tetramer exhibits unique properties for the small GTPase Rab5. *J. Biol. Chem.* **277**, 3412–3418
23. Kajiho, H., Saito, K., Tsujita, K., Kontani, K., Araki, Y., Kurosu, H., and Katada, T. (2003) RIN3: a novel Rab5 GEF interacting with amphiphysin II involved in the early endocytic pathway. *J. Cell Sci.* **116**, 4159–4168
24. Tabancay, A.P., Jr., Gau, C.L., Machado, I.M., Uhlmann, E.J., Gutmann, D.H., Guo, L., and Tamanoi, F. (2003) Identification of dominant negative mutants of Rheb GTPase and their use to implicate the involvement of human Rheb in the activation of p70S6K. *J. Biol. Chem.* **278**, 39921–39930
25. Bucci, C., Thomsen, P., Nicoziani, P., McCarthy, J., and van Deurs, B. (2000) Rab7: a key to lysosome biogenesis. *Mol. Biol. Cell* **11**, 467–480
26. Gutierrez, M., Munafo, D., Beron, W., and Colombo, M. (2004) Rab7 is required for the normal progression of the autophagic pathway in mammalian cells. *J. Cell Sci.* **117**, 2687–2697
27. Gozuacik, D. and Kimchi, A. (2004) Autophagy as a cell death and tumor suppressor mechanism. *Oncogene* **23**, 2891–2906
28. Kamada, Y., Sekito, T., and Ohsumi, Y. (2004) Autophagy in yeast: a TOR-mediated response to nutrient starvation. *Curr. Top. Microbiol. Immunol.* **279**, 73–84
29. Simonsen, A., Wurmser, A.E., Emr, S.D., and Stenmark, H. (2001) The role of phosphoinositides in membrane transport. *Curr. Opin. Cell Biol.* **13**, 485–492
30. Clark, G.J., Kinch, M.S., Rogers-Graham, K., Sebti, S.M., Hamilton, A.D., and Der, C.J. (1997) The Ras-related protein Rheb is farnesylated and antagonizes Ras signaling and transformation. *J. Biol. Chem.* **272**, 10608–10615
31. Yee, W.M. and Worley, P.F. (1997) Rheb interacts with Raf-1 kinase and may function to integrate growth factor- and protein kinase A-dependent signals. *Mol. Cell. Biol.* **17**, 921–933
32. Im, E., von Lintig, F.C., Chen, J., Zhuang, S., Qui, W., Chowdhury, S., Worley, P.F., Boss, G.R., and Pilz, R.B. (2002) Rheb is in a high activation state and inhibits B-Raf kinase in mammalian cells. *Oncogene* **21**, 6356–6365s
33. Karbowniczek, M., Cash, T., Cheung, M., Robertson, G.P., Astrinidis, A., and Henske, E.P. (2004) Regulation of B-Raf kinase activity by tuberin and Rheb is mammalian target of rapamycin (mTOR)-independent. *J. Biol. Chem.* **279**, 29930–29937
34. Pan, D., Dong, J., Zhang, Y., and Gao, X. (2004) Tuberous sclerosis complex: from *Drosophila* to human disease. *Trends Cell Biol.* **14**, 78–85
35. Aspuria, P. and Tamanoi, F. (2004) The Rheb family of GTP-binding proteins. *Cell Signal.* **16**, 1105–1112