Two Ras-related proteins, ERas and Rheb, which are involved in the phosphatidylinositol 3-kinase pathway, display high GTP affinity and have atypical CAAX motifs. The factors governing the intracellular localization of ERas and Rheb are incompletely understood. In the current study, we show by confocal microscopy that ERas is localized to the plasma membrane, whereas Rheb is confined to the endomembranes. Membrane localization of the two proteins was abolished by mutation of the cysteine of the CAAX motif. Membrane targeting was also abolished by a farnesyltransferase inhibitor but not by a geranylgeranyltransferase inhibitor. In mouse fibroblasts deficient in either Rce1 (Ras converting enzyme 1) or Icmt (isoprenylcysteine carboxyl methyltransferase), ERas was mislocalized mainly to the Golgi apparatus, whereas Rheb showed diffuse localization. Mutation of cysteines in the hypervariable region of ERas prevented the plasma membrane localization of ERas, very strongly suggesting that palmitoylation of the cysteines is essential for membrane targeting. The hypervariable region of Rheb does not contain cysteines or polybasic residues, and when it was replaced with the hypervariable region of H-Ras, Rheb displayed plasma membrane localization. These data indicate that ERas shares the same posttranslational modifications with H-Ras and N-Ras and is localized at the plasma membrane. Rheb also shares the same membrane-targeting pathway but because of the absence of palmitoylation is located on endomembranes.

The Ras protooncogenes consist of three closely related proteins: H-Ras, N-Ras, and K-Ras (1). Two alternatively spliced forms of K-Ras exist, K-RasA and K-RasB. Each of the four Ras proteins is localized at the plasma membrane. The determinants of plasma membrane localization, which is essential for Ras function, have been studied extensively (2). A sequence motif at the carboxyl terminus, the so-called CAAX motif (in which “C” is cysteine, “A” is generally an aliphatic amino acid, and X can be almost any amino acid), triggers isoprenylation of the cysteine residue by a cytosolic protein prenyltransferase. In the case of the Ras proteins, they are farnesylated by protein farnesyltransferase. Interfering with this lipid modification prevents plasma membrane localization (3). Initially, it was thought that prenylated Ras proteins were transported directly to the plasma membrane (4). However, it is now well established that the Ras proteins first move to the endoplasmic reticulum, where the last three amino acids of the CAAX motif (i.e. the -AA(X) motif) are released by an endoprotease, followed by methylation of the newly exposed prenylcyteine (5). These “postisoprenylation” modifications are believed to render the carboxyl terminus of the protein more hydrophobic, enhancing membrane binding. This was confirmed by the finding that inactivation of either Rce1 (Ras and a-factor converting enzyme 1), which releases the -AA(X), or Icmt (isoprenylcysteine carboxyl methyltransferase), which methylates the farnesylcyteine, impairs membrane association of H-Ras and K-RasB (6–8).

To ensure plasma membrane localization of H-Ras and N-Ras, another lipid modification, palmitoylation, is required (9). Palmitoylation occurs at one or two cysteine residues in the hypervariable region (HVR) upstream of the CAAX motif. Interfering with palmitoylation impairs plasma membrane localization of H-Ras. Thus, the combination of four posttranslational modifications, farnesylation, endoproteolysis, methylation, and palmitoylation is required for the plasma membrane localization of H-Ras and N-Ras. K-Ras4B does not contain the upstream cysteines and therefore cannot be palmitoylated (9). Instead, its sequence contains multiple lysine residues upstream of the CAAX motif, and that polybasic domain is very important for the plasma membrane localization of K-Ras4B.

In addition to H-Ras, N-Ras, and K-Ras, there are many Ras-related proteins, for example ERas (10) and Rheb (11). ERas (embryonic stem cell-expressed Ras) was identified by in silico analyses of the expressed sequence tag data bases to be a protein found specifically in mouse embryonic stem cells. ERas has less than 50% amino acid identity with H-Ras, but the key domains essential for Ras function are highly conserved (12). Rheb (Ras homolog expressed in brain) was originally identified as a brain-specific protein but is now known to be produced in many tissues (11). It has ~30% amino acid identity with H-Ras.

ERas and Rheb are only ~30% identical between at the amino acid sequence level, but they share several common functions. First, both are involved in the phosphatidylinositol 3-kinase signaling pathway. ERas directly binds to and activates the catalytic subunit (p110) of PI 3-kinase (10). Rheb is now known to function as a link between PI 3-kinase and the serine-threonine kinase mTOR (13–17).

Another similarity between ERas and Rheb is that both show high affinity to GTP (10, 18), a consequence of their unique amino acid

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sequences. In H-Ras, K-Ras, and N-Ras, valine 12 is highly conserved and is essential for the intrinsic GTPase activity (19). Mutation of valine 12 to any other amino acid residue lowers GTPase activity, thereby increasing the affinity for GTP and rendering the protein constitutively active and oncogenic. Both ERas and Rheb have different amino acids at the corresponding amino acid residue. ERas also contains other unique amino acid sequences promoting high GTP affinity.

The third similarity between ERas and Rheb is that they both have atypical carboxyl-terminal sequences (Fig. 1). In contrast to other Ras family proteins that terminate with typical CAA(-like) motifs, ERas also contains other unique amino acid sequences promoting high GTP affinity.

In this study, we studied intracellular localization of enhanced green fluorescence protein (EGFP)-tagged ERas and Rheb by confocal microscopy. By utilizing knock-out cells and specific inhibitors of enzymes involved in the posttranslational modifications, we examined mechanisms of membrane localization of the two proteins.

**Materials and Methods**

**Plasmid Construction.—**All of the cDNAs used in this study were amplified by reverse transcription-PCR and subcloned into pDONR201 or pENTR-D-TOPO (Invitrogen) except H-RasV12. Primer sequences used for reverse transcription-PCR were as follows: for K-Ras4B, Rap1A, and Rheb, CAA CAA AGC CAT ACA CCA AGC CAC CA-3'); and for ERas, SSEKTRH QAKV CGG CGA (pENTR-1A (Invitrogen)).

**Eras-SSVA-AS (5' TCA AGG TAC AGA GGA GCC ACA CCT GCA ACT-3'), H-Ras-SVLS-AS (5' TCA GGA GAC CAC AGA CTT GCA GCT-3'), and Rheb-SSVM-AS (5' TCA CAT CAC CCA AGA AGA CTT GCC TCC TCC-3') were used, respectively. EH-chimera-S (5' TGT CCA GAT TCA ACA CCA GCT CGG AAA GGC-3') and ERas-chimera-AS (5' AAT CTC ATG GAG ACAG GGC GGC AAA-3') were used for generating ERas-HVRRas. HE-chimera-S (5' TGC GTG GAG ATC CAG AGG GCC CAG GAC-3') and H-Ras-chimera-AS (5' CCG GAT CTC AGC CAG CAA CTA GCT-3') were used for generating H-RasV12-HVRRas.

To construct pMX-EGFP-Ip, an EcoRI fragment of pCX-EGFP (20) was introduced into the EcoRI site of pMX-IP. To construct pMX-EGFP-gw-Ip, an EcoRI/HindIII fragment of pEGFP-gw was introduced into the EcoRI/BamHI sites of pMX-IP. To construct pPyCAG-Myc-gw-Ip, a Clal/Xhol fragment of pCS2+MT-gw (21) was introduced into the Xhol site of pPyCAG-Ip.

**Cell Culture—**Wild-type immortalized mouse embryonic fibroblasts, fibroblasts homozygous for an Rce1 knock-out allele (8), and fibroblasts homozygous for an Icmt knock-out allele (6) were cultured in Dulbecco's modified Eagle's medium (Nacalai tesque) containing 10% fetal bovine serum (Bovest) and 50 units of penicillin/streptomycin (Invitrogen). MG1.19 embryonic stem cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 1 x 10^-4 M nonessential amino acids (Invitrogen), 2 mM L-glutamine (Invitrogen), 1 x 10^-3 M 2-mercaptoethanol (Invitrogen), and 50 units of penicillin/streptomycin (Invitrogen) plus leukemia inhibitory factor. PLAT-E packaging cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 50 units of penicillin/streptomycin supplemented with 1 µg/ml of puromycin (Sigma) and 100 µg/ml of blasticidin S (Funakoshi). Retroviral-mediated transfection was performed as previously described (10).

**Immunoblotting—**Preparation of cell lysates and Western blot analyses were performed as described (10). Dilution rate of primary antibodies was as follows: anti-GFP (1:1000; MBL), anti-phospho-Ser473-AKT (1:1000; Cell Signaling Technology), anti-AKT (1:1000; Cell Signaling Technology), anti-Myc (1:600; Santa Cruz Biotechnology), and anti-CDK4 (1:200; Santa Cruz Biotechnology). Detection was performed with ECL (Amersham Biosciences) reagents and a LAS3000 imaging system (Fuji).

**Subcellular Fractionation—**Cytosplamic and membrane fractions were prepared as described by Cox et al (22). The cells were washed once with PBS, collected in 1 ml of PBS, and transferred to microcentrifuge tubes. These cell suspensions were centrifuged at 10,000 rpm for 30 s. The pellets were resuspended in 1225 µl of buffer A (10 mM Tris-HCl, pH 7.5, 1 mM MgCl2, and protease inhibitors) and incubated on ice for 10 min. These cells were disrupted with a Dounce tissue homogenizer (20 strokes in pestle B; Wheaton) and then mixed with 225 µl of 1 M NaCl. A total of 450 µl was transferred to new microcentrifuge tubes as total lysate (T). To separate the crude membrane fraction (P100) from the cytosolic fraction (S100), the remaining homogenized sample (1000 µl) was transferred into a polycarbonate tube (TLS-55; Beckman) and centrifuged at 38,000 rpm (100,000 × g) for 30 min at 4°C in a swinging bucket rotor (TLS-55) in a tabletop ultracentrifuge. All of the supernatant fluid was collected into a new tube (S100), and the pellet was resuspended with 850 µl of buffer A and 150 µl of 1 M NaCl (P100). One tenth volume of 10× Hi-SDS RIPA buffer (50% SDS, 10% Nonidet-P 40, and 10% deoxycholate) was added to total lysate, S100, and P100 samples, and the samples were then incubated on ice for 10 min. These samples were then centrifuged at 15,000 rpm for 30 min, and the supernatant fluids were transferred to new microcentrifuge tubes. SDS-PAGE and immunoblotting were then performed.
**Membrane Localization of ERas and Rheb**

**FIGURE 2. The CAAX motifs of ERas and H-RasV12 are essential for plasma membrane localization.** EGFP-tagged ERas, ERas-SSVA, ERas-ΔCAAX, H-RasV12, H-RasV12-SVLS, or H-RasV12-ΔCAAX were introduced into MEFs by retroviral infection. Fluorescence was observed with a confocal microscope. Bars, 10 μm.

**FIGURE 3. Plasma membrane localization of ERas is dependent on farnesylation.** MEFs expressing EGFP, EGFP-ERas, EGFP-H-RasV12, EGFP-K-Ras4BV12, or EGFP-Rap1A were treated with dimethyl sulfoxide (DMSO), 2 μM of FTI-277, or 5 μM of GGTI-298 for 24 h. After treatment, the cells were fixed and observed with a confocal microscope. Bars, 10 μm.

Immunochemistry—The cells were seeded on coverslips and incubated overnight. On the next day, the coverslips were washed three times for 5 min with PBS. The cells were fixed with PBS containing 4% formaldehyde for 5 min at room temperature. After fixation, the coverslips were washed three times for 10 min with PBS and permeabilized with PBS containing 0.5% Triton X-100 for 10 min at room temperature. The coverslips were washed three times for 10 min with PBS, placed in blocking solution (PBS containing 1% bovine serum albumin) for 30 min at room temperature, and washed three times with PBS for 15 min. The coverslips were incubated with anti-GM130 antibody (diluted 1:200 in blocking buffer) for 1 h at room temperature and washed three times for 15 min with PBS. The coverslips were incubated with cyanine 3-labeled anti-mouse IgG (Chemicon) diluted 1:100 in blocking buffer for 1 h at room temperature, and washed three times for 10 min with PBS. The coverslips were incubated with anti-GM130 antibody (diluted 1:200 in blocking buffer) for 1 h at room temperature and washed three times for 15 min with PBS. The coverslips were incubated with cyanine 3-labeled anti-mouse IgG (Chemicon) diluted 1:100 in blocking buffer for 1 h at room temperature, and washed three times for 10 min with PBS. The coverslips were incubated with cyanine 3-labeled anti-mouse IgG (Chemicon) diluted 1:100 in blocking buffer for 1 h at room temperature, and washed three times for 10 min with PBS.

RESULTS

The CSVA Motif Is Essential for Plasma Membrane Localization of ERas—To examine the intracellular localization of ERas and the role of the carboxyl-terminal CSVA motif, we constructed an ERas-SSVA plasmid, in which cysteine in the CSVA motif was changed to serine. We also generated an H-RasV12-SVLS construct. In MEFs, both EGFP-ERas and EGFP-H-RasV12 were located at the plasma membrane. In contrast, EGFP-ERas-SSVA and EGFP-H-RasV12-SVLS were diffusely localized within the cytoplasm and the nucleus (Fig. 2). We also made mutants of ERas and H-RasV12, in which the last four amino acids were deleted but the hypervariable region was retained. These deletion mutants also displayed a diffuse cytoplasmic and nuclear localization pattern (Fig. 2). These data demonstrated that the CSVA motif is required for plasma membrane targeting of ERas.

ERas Is Farnesylated—To define whether ERas is farnesylated or geranylgeranylated, we generated MEFs stably expressing EGFP, EGFP-ERas, EGFP-H-RasV12, EGFP-K-Ras4BV12, or EGFP-Rap1A. We treated these cells with the 2 μM of the farnesytransferase inhibitor FTI-277 (3) or 5 μM of the geranylgeranytransferase inhibitor GGTI-298 (23) for 24 h. It has been shown that K-Ras4B can be either farnesylated or geranylgeranylated (24). When treated with Me3SO (vehicle), EGFP-ERas, EGFP-H-RasV12, and EGFP-K-Ras4BV12 were located at the plasma membrane (Fig. 3). Rap1A was located at the plasma membrane and in the cytoplasm but not in the nucleus. The cytoplasmic fluorescence was likely derived from endomembrane localization (Golgi apparatus and endoplasmic reticulum), as has been previously reported (25). When treated with FTI-277, the majority of EGFP-ERas and EGFP-H-RasV12 showed diffuse localization in both cytoplasm and nucleus. Signals in the nucleus indicate that the EGFP-Ras proteins can go through nuclear pores. In contrast, the localization of EGFP-K-Ras4BV12 and EGFP-Rap1A was unaffected. When treated with GGTI-298, the localization of EGFP-ERas, EGFP-H-RasV12, and EGFP-K-Ras4BV12 was not affected, whereas EGFP-Rap1A developed diffuse localization. These data indicate that farnesylation is required for membrane targeting of ERas.

Rce1 and Icmt Are Required for Translocation of ERas from the Golgi to the Plasma Membrane—To determine whether ERas requires Rce1 and Icmt for plasma membrane localization, we took advantage of the existence of MEFs deficient in these enzymes. The plasma membrane localization of EGFP-ERas, EGFP-H-RasV12, and EGFP-K-Ras4BV12 was partially blocked in both Rce1- and Icmt-deficient fibroblasts, suggesting that the two enzymes are required to achieve complete targeting of ERas to the plasma membrane (Fig. 4).

To quantify the inhibition of plasma membrane localization by Rce1 or Icmt deficiency, we performed subcellular fractionation and immunoblotting (Fig. 5). In wild-type cells, most of EGFP-ERas, EGFP-H-RasV12, and EGFP-K-Ras4BV12 was detected in the pellet (the P100-insoluble fraction). EGFP and EGFP-ERas ΔC proteins were detected in the soluble (S100) fraction. In Rce1- or in Icmt-deficient cells, by contrast, ~50% of the EGFP-ERas, EGFP-H-RasV12, and EGFP-K-Ras4BV12 was located in the P100 fractions, and ~50% was located in the S100 fractions.

We found that a significant portion of EGFP-ERas was condensed around the nucleus in Rce1- or Icmt-deficient cells. This pattern was suggestive of Golgi localization. To test this possibility, we stained these cells with an antibody against the Golgi matrix protein of 130 kDa (GM130) (26). Indeed, the condensed EGFP-ERas protein around the...
nucleus colocalized with GM130 in the cells lacking Rce1 or Icmt (Fig. 6). In contrast, EGFP-H-RasV12 was scarcely "merged" with GM130 in Rce1- or Icmt-deficient cells.

Two Cysteine Residues in the Hypervariable Region of ERas Are Required for Membrane Localization—Both ERas and H-Ras have two cysteine residues in the hypervariable region. We hypothesized that ERas was palmitoylated and that this modification might be required for the plasma membrane targeting of ERas. To confirm this hypothesis, we constructed ERas-C220S/C222S mutants, in which the two upstream cysteine residues were mutated to serines. We also constructed H-RasV12-C181S/C184S, in which the two palmitoylation sites were altered. These mutants were introduced into wild-type, Rce1-deficient, or Icmt-deficient MEFs (Fig. 7). EGFP-H-RasV12-C181S/C184S was detected in cytoplasm in wild-type MEFs but not at the plasma membrane or in the nucleus. It is likely that EGFP-H-RasV12-C181S/C184S was located in endomembranes, as has been previously reported (5). In Rce1- or Icmt-deficient cells, we observed a diffuse pattern in both the cytoplasm and the nucleus. In contrast, EGFP-ERas-C220S/C222S did not localize to endomembranes and showed a diffuse expression pattern, even in wild-type MEFs. These data indicate that the palmitoylation of the upstream cysteine residues in ERas is required for both plasma membrane and endomembrane localization, in contrast to the situation with H-Ras, where palmitoylation is required only for plasma membrane localization. Plasma membrane localization of ERas was also inhibited by the specific palmitoylation inhibitor 2-bromopalmitate (data not shown).

To examine mechanisms underlying the different localizations of EGFP-ERas-C220S/C222S and EGFP-H-RasV12-C181S/C184S, we exchanged the CSVA sequence of ERas with the CVLS sequence of H-RasV12 (Fig. 8). We found that the exchange of the last four amino acids did not alter the localization patterns of EGFP-ERas-C220S/C222S and EGFP-H-RasV12-C181S/C184S. We next exchanged the hypervariable region between EGFP-ERas-C220S/C222S and EGFP-H-RasV12-C181S/C184S. We found that EGFP-ERas-HVRH-Ras-C220S/C222S (containing the HVR of H-Ras) was located in endomembranes, whereas EGFP-H-RasV12-HVRERas-C181S/C184S (containing the HVR of ERas) showed diffuse localization (Fig. 8), indicating that the hypervariable region is responsible for the different localizations of ERas and H-Ras that are deficient in palmitoylation.

Rheb Is Located in Endomembranes and Not at the Plasma Membrane—We studied the intracellular localization of Rheb by expressing EGFP-Rheb fusion proteins in MEFs. In contrast to ERas and H-Ras,
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EGFP-Rheb showed granule-like fluorescence pattern in cytoplasm but not at the plasma membrane or in the nucleus (Fig. 9A). This pattern is similar to that of palmitoylation-deficient H-Ras, indicating that Rheb is located in endomembranes. When the hypervariable region of Rheb was replaced with that of H-Ras, the protein (Rheb-HVRH11-12a) appeared at the plasma membrane, suggesting that the lack of cysteines in its hypervariable region underlies its endomembrane localization. Mutation of the CSVM motif to SSVM resulted in diffuse localization of EGFP-Rheb.

The endomembrane localization of Rheb was also abolished when cells were treated with the farnesyltransferase inhibitor FTI-277 but not when the cells were treated with the geranylgeranyltransferase inhibitor GGTI-298 (Fig. 9B). In Rce1- or Icmt-deficient cells, EGFP-Rheb showed diffuse localization in both the cytoplasm and nucleus (Fig. 9C). These data suggest that posttranslational modifications by farnesyltransferase, Rce1, and Icmt are important for the transport of Rheb to endomembranes. However, Rheb cannot reach the plasma membrane because of the absence of either the palmitoylation sites or the polybasic motif.

Functional Relevance of Membrane Localization of ERas—Next, we studied effects of membrane localization on ERas functions. We utilized two ERas mutants, ERas-HVRH11-12a-C220S/C222S (which was located in endomembranes) and ERas-SSVA (which manifested diffuse localization). We first examined GTP/GDP association of wild-type ERas and the two mutant ERas proteins by thin layer chromatography. We found that more than 80% of ERas was associated with GTP regardless of localization (Fig. 10A). In addition, we found that ~50% of Rheb was associated with GTP regardless of localization. We then examined the activity of downstream effectors of ERas. Western blot analyses with specific antibodies showed that both the plasma membrane- and endomembrane- localized ERas increased phosphorylation of AKT (Fig. 10B). The plasma membrane-associated ERas was significantly more potent than endomembrane-associated protein. By contrast, nonmembrane-associated ERas failed to activate the PI 3-kinase pathway, despite high GTP association.

DISCUSSION

In this study, we examined the molecular mechanisms determining the membrane localization pattern for two Ras-related proteins, ERas and Rheb. The CAAAX motifs of these two proteins are atypical in that they have a serine at the A’ position, suggesting that they may be processed differently and have unique membrane targeting properties. We found, however, that ERas shares the same posttranslational modifications promoting plasma membrane localization as H-Ras and N-Ras.

The modifications include farnesylation of the cysteine in the CSVVA motif, release of the SVA triplet by Rce1, carboxymethylation of the farnesylcysteine by Icmt, and palmitoylation of the upstream cysteines in the hypervariable region. In addition to human and mouse proteins,
ERas orthologs have been identified in genomic data bases of rat, cow, and dog. The CSAV motif and the two cysteines in the HVR are conserved in all species, suggesting common plasma membrane-targeting mechanisms.

The effects of eliminating methylation, endoproteolysis (i.e. the -SVA release), or palmitoylation were not identical in H-Ras and ERas. In lcamt- or Rec1-deficient fibroblasts, a portion of the H-Ras still reached the plasma membrane. In contrast, most of the ERas localized in the Golgi apparatus in those cells. When palmitoylation was abrogated by mutating the upstream cysteines, H-Ras localized to the endomembranes, whereas ERas showed diffuse localization in both the cytoplasm and the nucleus. These data indicate that ERas depends more on methylation and palmitoylation for membrane localization than does H-Ras.

We showed that most ERas exists in the GTP-bound form regardless of membrane localization. Because ERas contains three amino acids corresponding to oncogenic and constitutively active Ras mutants (10), it is not affected by GTP-GDP exchange factors or GTPase-activating proteins. This situation is in contrast to H-Ras and Rap1A, where specific GTPase-activating proteins reside at the plasma membrane and endomembranes, respectively (27).

We also showed that membrane localization of ERas is essential for activation of the PI 3-kinase pathway. This is reasonable because PI 3-kinase phosphorylates membrane-associated phospholipids. However, the reason that plasma membrane-associated ERas was more effective than endomembrane-associated ERas is not clear. The downstream effector Akt and/or its kinase PKD1 might be more accessible in the plasma membrane than in the endomembranes. Further studies are required to clarify this issue.

Another important finding of our study was that Rheb is localized to endomembranes and was not found at the plasma membrane. Endomembrane localization was blocked by a farnsyltransferase inhibitor but not by a geranylgeranyltransferase inhibitor. Endomembrane localization also disappeared in lcamt- or Rec1-deficient cells. These indicate that Rheb utilizes the same membrane targeting machinery as H-Ras and ERas.

Why is Rheb targeted to endomembranes? The localization pattern is similar to that of Rap1A (28), in which the CAAX motif (CLL) is geranylgeranylated. In addition, the hypervariable region of Rap1A contains a polybasic stretch similar to K-Ras4B, indicating that it should be located at the plasma membrane. However, a domain within Rap1A (spanning amino acids 85–89) prevents translocation to the plasma membrane (28). In contrast, Rheb lacks the upstream cysteines or polybasic stretches in the hypervariable region. When we inserted the H-Ras hypervariable region into Rheb (Rheb-HVR-H-Ras), we found that the protein was targeted to the plasma membrane. Thus, the lack of palmitoylation or polybasic residues is responsible for the endomembrane localization of Rheb.

Although several studies demonstrated that farnesylation is essential for Rheb function, its intracellular localization has not been fully elucidated. Clark et al. (29) performed immunohistochemistry on cells transfected with HA-tagged Rheb and H-Ras and concluded that the two proteins displayed similar plasma membrane localization. However, they did not use confocal microscopy, which facilitates precise determination of intracellular localization. Our data clearly show that EGFP-Rheb is located on endomembranes, which is entirely reasonable because Rheb lacks the upstream cysteines or polybasic domains in the HVR.

Rheb is conserved through evolution. Its orthologs have been identified in yeast, fungus, worm, fish, frog, and chicken (30). In addition, a mammalian paralog (designated Rheb1 or Rheb2) sharing 50% amino acid identity with Rheb was identified (30). These Rheb proteins all have CAAX motifs. By contrast, none of them contains cysteines or polybasic residues in the HVR. Therefore, it is likely that endomembrane localization is common among all Rheb proteins.

What is the functional relevance of the endomembrane localization of Rheb? One possibility is that it facilitates association with downstream effectors. Several studies have suggested that mTOR functions downstream of Rheb (15). The CSVM motif and farnesylation have been shown to be essential for activation of mTOR (18, 29, 31). Because mTOR exists in the Golgi and endoplasmic reticulum (32), endomembrane localization of Rheb should provide better access to the target effector.

Another possibility is that endomembrane localization ensures proper regulation of Rheb activity. It has been shown that TSC1 and TSC2 form a heterodimer and function as a GTPase-activating protein (16, 17). We found that an EGFP-TSC1 fusion was located in endomembranes in MEFs (data not shown). The CAAX motif of Rheb might be required for efficient interaction with the TSC1-TSC2 complex and for proper regulation. In conclusion, we show that two Ras-related proteins, ERas and Rheb, activate the PI 3-kinase pathway from different membrane compartments. This finding adds new examples to the emerging concept that subcellular compartments play pivotal roles in intracellular signaling by many molecules, including Ras and other small GTPase proteins.

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