Small GTPase Rah/Rab34 Is Associated with Membrane Ruffles and Macropinosomes and Promotes Macropinosome Formation*

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Running title:
Rah Promotes Macropinosome Formation

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SUMMARY

Macropinocytosis is an efficient way for the uptake of nutrients and solute macromolecules into cells from the external environment. Macropinosomes, which are surrounded by actin, are formed from the cell surface membrane ruffles and migrate toward the cell center. We have cloned the entire coding sequence of a member of the Rab family small GTPases, Rah/Rab34. It lacked a consensus sequence for GTP-binding/GTPase domain. Although wild-type Rah exhibited extremely low GTPase activity in vitro, it exerted appreciable GTPase activity in vivo. In fibroblasts, Rah was colocalized with actin to the membrane ruffles and membranes of relatively large vesicles adjacent to the ruffles. These vesicles were identified as macropinosomes on the basis of several criteria. Rah and Rab5 coexisted in some, but not all, macropinosomes. Rah was predominantly associated with nascent macropinosomes, whereas Rab5 was present in endosomes at later stages. The number of macropinosomes in the cells overexpressing Rah increased about twofold. The formation of macropinosomes by the treatment of platelet-derived growth factor or phorbol ester was also facilitated by Rah but suppressed by a dominant-negative Rah. Rah-promoted macropinosome formation was retarded by dominant-negative mutants of Rac1 and WAVE2, which are essential for membrane ruffling. These results imply that Rah is required for efficient macropinosome formation from the membrane ruffles.
INTRODUCTION

Endocytosis in eukaryotic cells serves to maintain cellular and organismal homeostasis by taking up fluids and macromolecules, signaling molecules, and their receptors from the external environment (1). There are at least 5 independent endocytic processes: clathrin-dependent endocytosis mediated by clathrin-coated vesicles (100–150 nm in diameter), caveolin-dependent endocytosis mediated by caveolae (50–80 nm), clathrin- and caveolin-independent endocytosis, macropinocytosis, and phagocytosis (2, 3). Among these processes, macropinocytosis is carried out with relatively large vesicles (0.2–5 µm in diameter) formed from cell surface membrane ruffles folding back on the plasma membrane (4, 5). Macropinosomes are not coated with clathrin or caveolin but surrounded by actin at early stages. Macropinocytosis provides an efficient way for non-selective uptake of nutrients and solute macromolecules. It also accounts for internalization of extracellular antigens by professional antigen-presenting cells like dendritic cells. Furthermore, macropinocytosis is postulated to play important roles in chemotaxis by regulating plasma membrane–actin cytoskeleton interaction and membrane trafficking. Some pathogenic bacteria such as *Salmonella typhimurium* and *Shigella flexneri* also exploit macropinocytosis to invade the cells (6).

Treatment of various types of cultured cells with growth factors, cytokines, phorbol esters such as phorbol 12-myristate 13-acetate (PMA)¹, or diacylglycerol elicits a rapid and dramatic membrane ruffling and macropinocytic responses (7–13). Introduction of small GTPases, Ras or Rac1, or Tiam1, a guanine nucleotide exchange factor (GEF) for Rac1, also induces membrane ruffling and macropinocytosis in fibroblasts (13–15). The dominant-negative mutant Rac1(T17N) interferes with membrane ruffling and macropinosome formation induced by growth factors, PMA, Ras, or Tiam1 (13, 15). This implies that the ruffling and macropinosome formation by these agents are mediated by Rac1. Rac1 causes membrane ruffling through its target protein IRSp53, which activates WAVE2/Scar2 (16–18). WAVE2 is involved in the formation of branched actin filament meshwork in membrane ruffles by activating Arp2/3 complex (18–20).

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Rab family small GTPases play essential roles in the endocytic and exocytic pathways. It is well established that Rab proteins function in the targeting and docking of the vesicles to their acceptor membranes. However, it has become evident that at least some of them exert their functions in multiple steps of vesicular trafficking including vesicle formation, vesicle motility, membrane remodeling, and vesicle fusion as well as vesicle targeting and docking (21–24). In mammalian cells, >50 members of Rab family proteins have been identified. These proteins are associated with particular vesicle membrane compartments and function in specific stages of the diverse vesicle trafficking events. Among them, Rab5 is located to the membranes of clathrin-coated vesicles and early endosomes. It is involved in receptor-mediated endocytosis and fluid-phase pinocytosis (25–27). Introduction of a constitutively active Rab5 mutant in cells stimulates the rate of endocytosis and homotypic fusion of early endosomes, whereas its dominant-negative mutant prevents the vesicle fusion (26). Rab4 is also present in early endosome membrane and implicated in recycling pathway from endosomes to the plasma membrane (28). Rab7 is present in late endosome membrane and participates in transport from early to late endosomes and lysosomes (29, 30). Although a mutant Rab5 protein defective in GTP-binding ability retards fluid-phase pinocytosis in addition to receptor-mediated endocytosis (25), there has been no report indicating that any Rab family proteins are specifically located to the membrane ruffles or macropinosomes and play direct roles in macropinosome formation.

Rah is a small GTPase postulated to be a member of Rab family due to the similarity of its effector domain and C-terminal sequences to those of several Rab proteins (31). Since only a truncated cDNA lacking the sequence encoding N-terminal portion was available (31), we have cloned in the present study the cDNA containing the entire coding region of mouse Rah. Rah was colocalized with actin to the membrane ruffles and macropinosome membrane. During macropinosome biogenesis, Rah associated with nascent macropinosomes seemed to be replaced by Rab5 on the early endosomes. Overexpression of Rah elevated the number of macropinosomes, whereas the expression of a dominant-negative form of Rah prevented the formation of macropinosomes induced by platelet-derived growth factor (PDGF) or PMA. On the other hand, Rah-promoted macropinosome formation was retarded by dominant-negative
mutants of Rac1 and WAVE2. Thus, Rah is likely to play crucial roles in the formation of macropinosomes from the membrane ruffles.

EXPERIMENTAL PROCEDURES

Cloning of Rah and Rab5—Mouse heart total RNA was prepared according to Chomczynski and Sacchi (32), and poly(A)+ RNA was isolated from the total RNA with Oligotex-dT30 Super (Roche Diagnostics). A single-stranded cDNA pool was synthesized with SuperScript II RNase H(−) reverse transcriptase (Invitrogen) from the poly(A)+ RNA primed with an oligo(dT) primer. Rah cDNA fragment was cloned from the cDNA pool by reverse transcription-polymerase chain reaction (RT-PCR) with Taq DNA polymerase (Qiagen) by using a sense primer 5′-AAGGTCACTGTTGTGGAGA-3′ (nucleotides 7–26 of the DDBJ/EMBL/GenBank accession number S72304) and an antisense primer 5′-GGGACAACATGTGGGCTTTT-3′ (nucleotides 605–624). The cDNA library of the mouse skeletal muscle cell line C2 myoblasts constructed in λZAPII (33) was screened with the PCR product. Rab5b cDNA containing the entire coding region was cloned from the heart cDNA pool by RT-PCR using a sense primer 5′-GGAGGACATATGACTAGCAG-3′ (nucleotides 42–61 of the accession number X84239) and an antisense primer 5′-CACCCCTCAGTTGCTACAAC-3′ (nucleotides 685–704). Mouse H-Ras and Rac1 cDNAs were cloned as described previously (33). Nucleotide sequence of the cloned cDNAs was determined with LI-COR 4000 automated DNA sequencing system by use of SequiTherm Long-Read Cycle Sequencing Kit-LC (Epicentre Technologies). The nucleotide and amino acid sequences were analyzed with GENETYX-Mac softwares (Ver. 10.1, Software Development Co.).

Expression and Purification of Recombinant Proteins—Point mutations to generate Rah(Q111L) and Rah(T66N) analogous to a constitutively active form of Ras(Q61L) and a dominant-negative form of Ras(S17N), respectively, were introduced in Rah cDNA with Transformer site-directed mutagenesis kit (Clontech). Similarly, point mutations to generate
constitutively active Rab5(Q79L) and dominant-negative Rab5(S34N) were introduced in Rab5 cDNA. Coding sequences of the wild-type (wt) or the mutated Rah and Rab5 were fused in frame to glutathione-S-transferase (GST) in pGEX-2T (Amersham). These recombinant fusion proteins were expressed in E. coli strain XL1-Blue and affinity-purified with glutathione-Sepharose (Amersham) as described (34).

**GTP-binding/GTPase Assay**—*In vitro* GTP-binding/GTPase assay of the recombinant GST-fusion proteins by thin-layer chromatography was carried out as described previously (33). Briefly, each GST-fusion protein bound to glutathione-Sepharose was incubated with 370 kBq of [α-32P]GTP (>111 TBq/mmol, ICN Biomedicals, Inc.) in the incubation buffer (50 mM NaCl, 50 mM Tris–HCl, pH 7.5, 5 mM EDTA, 0.1 mM EGTA, 0.1 mM dithiothreitol (DTT), and 10 µM ATP) at 37°C for 10 min. After washing with the wash buffer (20 mM MgCl₂, 50 mM Tris–HCl, pH 7.5, 1 mM DTT, and 1 mg/ml bovine serum albumin), GTPase reaction was carried out in the assay buffer (50 mM NaCl, 5 mM MgCl₂, 50 mM Tris–HCl, pH 7.5, 5 mM DTT, and 1 mM GTP) at 37°C for 30 min and stopped by the addition of the ice-cold wash buffer. After washing with the wash buffer, bound nucleotides were eluted by incubating the Sepharose beads in 1% SDS and 20 mM EDTA at 65°C for 5 min. Dissociated samples were spotted on polyethyleneimine-cellulose plates (Macherey-Nagel). The released nucleotides were resolved by thin-layer chromatography in 0.75 M KH₂PO₄ and visualized by autoradiography.

*In vivo* GTP-binding/GTPase assay was conducted essentially according to Muroya *et al.* (35). Entire coding region of the wt or the constitutively active Rah and Rab5 were fused in frame to the Myc-tag in pEF-BOS vector. COS-1 cells (36) cultured in Dulbecco’s-modified Eagle’s (DME) medium containing 10% fetal bovine serum (growth medium) were transfected with these plasmids by using FuGENE 6 transfection reagent (Roche). Twelve hours after the transfection, the medium was replaced with phosphate-free DME medium supplemented with 10% fetal bovine serum dialyzed against 0.15 M NaCl and 10 mM HEPES–NaOH (pH 7.9). Phosphorus-32 (³²Pi) (ICN Biomedicals, Inc.) was added to the medium at 9.25 M Bq/ml and incubated for further 12 h. The cells were lysed with the lysis
buffer (150 mM NaCl, 20 mM MgCl2, 50 mM Tris–HCl, pH 7.5, 0.5% Nonidet P-40, 1 mM Na3VO4, and 2 µg/ml aprotinin). The anti-Myc monoclonal antibody (mAb) Myc1-9E10 (37) (American Type Culture Collection) was added to the cell lysates and incubated at 4°C for 60 min, and then Protein G Sepharose 4 Fast Flow (Amersham) was mixed and incubated for further 60 min. After thorough washing with the lysis buffer, bound nucleotides were dissociated with the dissociation buffer (20 mM Tris–HCl, pH 7.5, 20 mM EDTA, 2% SDS, 0.5 mM GDP, and 0.5 mM GTP) at 65°C for 5 min. Four µl each of the dissociated samples were analyzed by thin-layer chromatography followed by autoradiography.

Epitope-tagging, EGFP-tagging, and Fluorescence Microscopy—Entire coding region of the wt or the mutated Rah and Rab5 were fused in frame to the Myc-tag in pCMVmyc vector (33), to the hemagglutinin (HA)-tag in pEF-BOS vector, and to the enhanced green fluorescent protein (EGFP) sequence in pEGFP-C1 vector (Clontech). Human WAVE2 and its dominant-negative mutant WAVE2(ΔV) were fused to the FLAG-tag in pEF-BOS vector and to EGFP in pEGFP-C1 vector (38). The mouse C3H/10T1/2 (10T1/2) fibroblasts (39) cultured on glass coverslips in the growth medium were transfected with these plasmids by the calcium phosphate-mediated method as described (40). To detect macropinosomes, rhodamine B isothiocyanate (RITC)–dextran (mol. wt. ~70,000, Sigma) was added to the medium at the concentration of 1 mg/ml. To induce macropinocytosis, the cells were treated with 5 U/ml PDGF (Calbiochem) or 0.1 µM PMA (Sigma) for 30 min. The transiently transfected cells were processed for fluorescence microscopy 24 h after the transfection (40). The fixed and permeabilized cells transfected with Myc-, HA-, and FLAG-tagging constructs were incubated with the mAb Myc1-9E10, anti-HA rabbit polyclonal antibody (MBL), and anti-FLAG-tag mAb (Sigma), respectively. Then they were incubated with fluorescein isothiocyanate- or rhodamine-conjugated goat anti-mouse or anti-rabbit IgG (affinity-purified, Cappel). Actin filaments were detected by the staining with rhodamine–phalloidin (Molecular Probes, Inc.). Lamp1 was detected by the staining with mAb H4A3 (41) (Developmental Studies Hybridoma Bank) followed by the incubation with rhodamine–goat anti-mouse IgG. The specimens were observed with a Zeiss Axioskop microscope equipped with phase-
contrast and epifluorescence optics. The number of macropinosomes was estimated by counting relatively large vesicles (0.2–5 µm in diameter) surrounded by actin in >100 transfected cells in each transfection experiment.

*Time-lapse Microscopy*—Living 10T1/2 cells transfected with the pEGFP-C1 constructs were observed with a Zeiss Axiovert 135TV microscope equipped with phase-contrast and epifluorescence optics. The EGFP fluorescence was recorded through CoolSNAP digital CCD camera (Roper Scientific) and analyzed by IPLab/Mac imaging software (Scanalytics, Inc.).

**RESULTS**

**Sequence and Functional Domains of Rah**—As Rah cDNA available at that time had lacked the sequence corresponding to the N-terminal region (31), we tried to obtain a cDNA containing the entire coding region and cloned a 1.6-kb cDNA (accession number AB082927) from the mouse C2 skeletal muscle myoblast cDNA library. Because there was an in-frame termination codon (T416AA) upstream of A503TG in this clone, this ATG is most likely to serve the initiation codon. The coding region of the former truncated clone contains 208 amino acids (31), whereas that of this clone consisted of 259 amino acids and its calculated \( M_r \) was 29,128. As Rah was most similar to Rab family proteins among small GTPase families and its amino acid sequence was identical to that of mouse Rab34 (accession number BC038638, the data of which were submitted during revising this manuscript), we compared its amino acid sequence with those of several other Rab family proteins (Fig. 1A). Rah/Rab34 had the longest N-terminal sequence among Rab family proteins except for Rab36 (see Fig. 1B). Small GTPases generally contain conserved four sequence motifs for GTP/GDP-binding and GTPase activities (42, 43). Although Rah contained the first three motif sequences, it lacked the consensus sequence for the fourth motif (EXSA) (X, any amino acid) (Fig. 1A). Sequence of the putative effector domain of Rah was distinct from those of the other Rab
family proteins, suggesting that Rah exerts unique cellular functions. The C-termini of unprocessed Rab proteins usually end with -CC, -CCX<sub>1-3</sub> or -CXC, and the cysteine residues in these motifs are postulated to be geranylgeranylated (44). Since the C-terminal end of Rah was -CCP, it coincides with the motifs.

Rab36 is a recently identified Rab family protein encoded by the gene locating at 22q11.2, but its function has totally been unknown (45). It is the largest Rab family small GTPase to date consisting of 333 amino acids with extremely long N-terminal sequence (Fig. 1B). Rah was most closely related to Rab36, and the identity between the two proteins was 57% over the entire length of Rah (Fig. 1B). Even the long N-terminal portion of Rah showed a similarity to the corresponding region of Rab36. Notably, the sequences of their putative effector domains were identical, and in addition, sequences around the effector domains were also relatively well conserved between these two proteins. The fourth motif for GTP/GDP-binding was abrogated also in Rab36. The consensus sequence for phosphorylation by tyrosine kinases ((R/K)Y<sub>2-3</sub>(D/E)Y<sub>2-3</sub>) was present in Rah as well as in Rab36. This putatively phosphorylatable tyrosine falls in with the first amino acid in the effector domain. This might suggest that binding of target proteins to Rah and Rab36 is regulated by the tyrosine phosphorylation.

**Rah Exerts Low GTPase Activity in Vitro but Substantial Activity in Vivo**—Small GTPases basically exhibit GTP/GDP-binding and intrinsic GTPase activities. We analyzed these activities of Rah and Rab5 in vitro by thin-layer chromatography. Point mutations were introduced in the cDNA to generate Rah(Q111L) and Rab(T66N), which are equivalent to a constitutively active form (a mutant deficient in GTP hydrolysis) of Ras(Q61L) or Rab5(Q79L) (26) and a dominant-negative form (a mutant stabilized in GDP-bound form) of Ras(S17N) or Rab5(S34N) (26), respectively. The GST-tagged proteins of the wt and these mutants expressed in *E. coli* were used for the analyses of the activities. Although Rab5(wt) easily hydrolyzed bound GTP into GDP, Rah(wt) hydrolyzed bound GTP much less efficiently (Fig. 2A). Rah(Q111L) as well as Rab5(Q79L) was deficient in GTP hydrolyzing activity. Rah(T66N) was lacking in GTP-binding ability as was Rab5(S34N). The low
intrinsic GTPase activity of Rah(wt) might be ascribable to the lack of the fourth motif for GTP/GDP-binding and GTPase activities.

We further analyzed these activities of Rah and Rab5 \textit{in vivo}. Myc-tagged wt and constitutively active mutants of Rah and Rab5 were transiently transfected to COS-1 cells. Each of these proteins labeled with $^{32}$Pi was immunoprecipitated with the anti-Myc mAb and the bound guanine nucleotides were analyzed by thin-layer chromatography. Rab5(wt) bound high amount of GDP and much less amount of GTP, whereas Rab5(Q79L) bound almost equivalent amount of GTP and GDP (Fig. 2B). Remarkably, Rah(wt) bound low amount of GTP and high amount of GDP as did Rab5(wt). Furthermore, Rah(Q111L) bound less amount of GTP than did Rab5(Q79L). These results imply that, in contrast with the low GTPase activity \textit{in vitro}, Rah(wt) exhibits considerable level of GTPase activity \textit{in vivo} comparable to the activity of Rab5(wt). They also indicate that even the constitutively active mutants exert appreciable GTPase activity \textit{in vivo}. This is presumably due to the presence of GTPase-activating protein (GAP) in cells. Indeed, GAP activity stimulates GTPase activity of the constitutively active Rab3A \textit{in vivo} (46). Thus, specific GAPs are likely to exert their functions not only on Rab5(wt) but also on Rah(wt) and both these constitutively active mutants \textit{in vivo}.

\textit{Rah Is Colocalized with Actin to Membrane Ruffles and Nascent Endosome Membranes}

To infer cellular functions of Rah, we first determined the localization of Rah. Myc-epitope-tagged or EGFP-tagged Rah and its point mutants were transiently expressed in mouse 10T1/2 fibroblasts by transfection. Myc-tagged wt Rah was predominantly located to the cell surface membrane ruffles and membranes of relatively large (usually several \( \mu \)m in diameter) vesicles adjacent to the ruffles (Fig. 3, \( a \) and \( b \)). Staining with rhodamine–phalloidin showed that actin coexisted with Rah in these membrane structures (Fig. 3, \( c \) and \( d \)). Myc–Rah(Q111L) was also located to these membrane structures, and the phenotype of Myc–Rah(Q111L)-expressing cells was indistinguishable from that of the Myc–Rah(wt)-expressing cells (Fig. 3, \( e–h \)). On the other hand, most of the Myc-tagged dominant-negative Rah(T66N) was diffusely distributed throughout the cytoplasm and also associated with
membrane ruffles but not with the vesicles, even if they existed (Fig. 3, i–l). EGFP-tagged Rah(wt) and the point mutants distributed in the cells similarly to each of the Myc-tagged proteins (data not shown).

Rab5 is located to the membranes of early endosomes, and expression of the constitutively active Rab5(Q79L) causes the formation of unusually large endosomes by the homotypic fusion of early endosomes (25, 26). To identify the Rah-associated vesicles, Myc- or EGFP-tagged Rab5(wt), Rab5(Q79L), and the dominant-negative Rab5(S34N) were expressed in 10T1/2 cells. Both Rab5(wt) and Rab5(Q79L) were localized to the early endosome membranes, and actin was associated with the membranes of only some but not all of the endosomes (Fig. 4A, a–f). The endosomes associated with actin seem to be macropinosomes and those without actin association are likely to be either receptor-mediated endosomes or macropinosomes at later stages. Expression of Rab5(Q79L) resulted in the formation of large endosomes as much as 10–20 µm in diameter (Fig. 4A, d–f). In contrast, Rab5(S34N) was apparently diffusely distributed throughout the cytoplasm (Fig. 4A, g–i), consistent with the former results (26). None of these Rab5 proteins were integrated into the membrane ruffles.

Coexpression of Myc–Rah and EGFP–Rab5(Q79L) showed that these two Rab family proteins coexisted in a subset of vesicular membranes (Fig. 4B). Furthermore, there were vesicles containing either Rah or Rab5(Q79L). The Rah-containing vesicles were present peripherally in the cells, whereas the Rab5-containing endosomes occupied more central areas of the cells. In these coexpressing cells, Rah but not Rab5 was associated with membrane ruffles. These results suggest that the Rah-associated vesicles are endosomes and that they are nascent endocytic vesicles preceding early endosomes or are a part of early endosomes.

To further analyze the temporal and spatial relationship between Rah- and Rab5-associated endosomes, we examined whether Rah and Rab5 were colocalized to the endosome membranes with Lamp1, a marker membrane protein for late endosomes and lysosomes (41). Cells transfected with EGFP–Rah or EGFP–Rab5(Q79L) were stained with the anti-Lamp1 mAb H4A3. The antibody detected small vesicles in the central areas of the cells but did not the Rah-associated peripheral endosomes (Fig. 5A, a–c). In Rab5(Q79L)-
expressing cells, some of the Rab5(Q79L)-associated enlarged vesicles were labeled by the anti-Lamp1 (Fig. 5A, d–f). In addition, enlarged vesicles that did not contain Rab5(Q79L) were also labeled by the antibody. These results corroborate the above postulation that Rah-associated vesicles are nascent endocytic vesicles or a part of early endosomes.

**Rah Is Associated with Macropinosome Membrane**—The vesicles where Rah is located are predicted to be macropinosomes among various types of endosomes because of their size, the location close to the membrane ruffles, and coexistence with actin (4). As fluorescent dextrans are used to detect fluid-phase pinocytosis (47), we added RITC-conjugated dextran to the culture medium of Rah- or Rab5(Q79L)-transfected 10T1/2 cells and incubated for 1 h. RITC–dextran was taken up in many of the Rah-associated vesicles (Fig. 5B, a–c). It was incorporated in some of the Rab5(Q79L)-associated enlarged vesicles as well (Fig. 5B, d–f).

Macropinosomes are formed from the membrane ruffles (4). To determine whether Rah-associated endosomes were formed from the membrane ruffles, we exploited time-lapse microscopy on the living cells expressing EGFP–Rah. In the EGFP–Rah-expressing cells, EGFP fluorescence was detected at the membrane ruffles (Fig. 6). Relatively large vesicles were generated at the membrane ruffles and they migrated away from the cell margin towards the cell center (Fig. 6). Taken together, these results imply that the Rah-associated vesicles are macropinosomes.

**Rah Facilitates the Formation of Macropinosomes**—Next we examined which step of macropinocytosis Rah is involved in, *i.e.*, in the formation of macropinosomes, in the movement of macropinosomes, or in any other steps. For convenience, relatively large vesicles coated by actin, which was recognized by the staining with rhodamine–phalloidin, were regarded as macropinosomes. When EGFP–Rah(wt) or EGFP–Rah(Q111L) was expressed in 10T1/2 cells, the number of actin-coated macropinosomes per cell increased more than twofold over a mock-transfected cell (Fig. 7A). On the other hand, expression of EGFP–Rah(T66N) resulted in a slight decrease in the number of macropinosomes per cell. Consequently, exogenously expressed Rah facilitates the macropinosome formation over the
control level, and intrinsic Rah may contribute to a limited degree to the control level formation. In contrast with the number of macropinosomes, the degree of membrane ruffling was not affected by the expression of any of these Rah proteins (see Fig. 3–6).

Treatment of diverse types of cultured cells with growth factors or PMA induces prominent membrane ruffling and subsequent macropinocytosis (7–9, 11–13). When 10T1/2 fibroblasts were treated with PDGF or PMA, membrane ruffling and macropinosome formation were facilitated (Fig. 7B). The number of macropinosomes per cell elevated about twofold over a control level. To elucidate the relationship between Rah-promoted macropinocytosis and PDGF- or PMA-induced macropinocytosis, EGFP–Rah(wt)-transfected cells were treated with PDGF or PMA. The number of macropinosomes per cell elevated more than threefold over the control level in either case (Fig. 7B). Next, EGFP–Rah(T66N)-transfected cells were treated with each of these reagents. Expression of the dominant-negative Rah highly reduced the number of actin-associated macropinosomes per cell both in the PDGF- and the PMA-treated cells, but the levels remained higher than the control level (Fig. 7B). These results indicate that Rah participates in the macropinosome formation synergistically to these reagents.

Introduction of Ras or Rac1 induces membrane ruffling and macropinocytosis (13, 14). WAVE2 activated by IRSp53, a target protein of Rac1, is responsible for the Arp2/3 complex-mediated formation of the branched actin filament meshwork in membrane ruffles (16–18, 20). In addition, Dictyostelium discoideum Scar, an ortholog of mammalian WAVE2, is required for macropinocytosis (48). Thus, we examined the relationship among Ras, Rac1, WAVE2, and Rah in macropinocytosis. The number of actin-associated macropinosomes induced by Rah was reduced to ~72% by the coexpression of the dominant-negative H-Ras(S17N) but the level was over the control level (Fig. 7C). In contrast, coexpression of the dominant-negative Rac1(T17N) resulted in remarkable reduction in the number of macropinosomes to ~8%. EGFP-tagged WAVE2 coexisted with Rah in the membrane ruffles and macropinosomes (Fig. 7D). When FLAG-tagged WAVE2(ΔV), a dominant-negative WAVE2 mutant lacking verprolin-homology domain (35), was coexpressed with Rah, the number of macropinosomes was declined close to the control level (Fig. 7C). Taken together,
these results imply that Rah facilitates the macropinosome formation triggered by Rac1–IRSp53–WAVE2-induced membrane ruffling and that Rac1–IRSp53–WAVE2-mediated actin polymerization and subsequent membrane ruffling are required for the Rah-promoted macropinosome formation.

**DISCUSSION**

We have cloned the entire coding sequence of Rah/Rab34. It consists of 259 amino acids, and its large size is ascribed to its long N-terminal sequence. Usually the N- and C-terminal sequences are diverged even among the same family of small GTPases. Rah was most closely related to Rab36, the largest Rab family protein, over its entire length and also in its N-terminus. Although roles of the N-terminal sequences of small GTPases have not been well understood, some small GTPases bind particular proteins at their N-termini. For instance, RalA interacts at its N-terminus with phospholipase D1 in collaboration with Arf1 (49, 50). Rnd1 and RhoE interfere with both Rho- and Rac-mediated reorganization of the actin cytoskeleton. Deletion of the N-terminal six amino acids in Rnd1, however, results in deprivation of the antagonistic effects on the cytoskeleton (51). In addition, Rnd2, which lacks the N-terminal extending sequence present in RhoE and Rnd1, has no observable effects on the cytoskeleton (51). Thus, the long N-terminal sequence of Rah seems to be involved in the interaction with some proteins to exert its cellular functions. The similarity between the N-terminus of Rah and the corresponding sequence of Rab36 raises the possibility that these proteins share the proteins interacting with these sequences. Furthermore, Rah and Rab36 exhibit the identity between their core effector domains and close similarity between their extended effector domains. This may imply that these proteins also share several target proteins.

Although small GTPases have four conserved motifs for GTP/GDP-binding and GTPase activities, Rah lacked the fourth motif. In addition, the fourth motif was abrogated in Rab36 as well. The low intrinsic GTPase activity of Rah(wt) in vitro might be ascribable to the lack
of this motif. However, Rah(wt) exhibited substantial GTPase activity in vivo comparable to the activity of Rab5(wt). In addition, even the constitutively active Rah(Q111L) and Rab5(Q79L) exerted appreciable GTPase activity in vivo. This is presumably due to the presence of specific GAP activities in cells as has been shown with Rab3A (46). Particularly, the GAP activity acting on Rah seems to be strong because Rah(Q111L) showed higher GTPase activity than did Rab5(Q79L) and almost comparable to that of Rah(wt) in vivo. Furthermore, certain functions including endocytic ability are indistinguishable between Rab5(wt) and Rab5(Q79L) (52, 53). This may explain why the phenotype of the cells transfected with Rah(wt) and that with Rah(Q111L) were indistinguishable.

Rah was colocalized with actin to the membrane ruffles and membranes of relatively large vesicles adjacent to the ruffles. In addition, time-lapse microscopy showed that the vesicles were actually formed from the membrane ruffles. These vesicles are identified as macropinosomes because of their large size, formation from the membrane ruffles, incorporation of dextran from the medium, and colocalization with actin. Although >50 members of Rab family proteins have been identified in mammalian cells (22–24), Rah is the first Rab family protein, to our knowledge, associated specifically with both the membrane ruffles and macropinosome membranes. Rab5 is located to the membranes of clathrin-coated vesicles and early endosomes. It participates in receptor-mediated endocytosis and fluid-phase pinocytosis (25–27). Coexpression of Rah and Rab5(Q79L) showed that Rah but not Rab5 was associated with membrane ruffles. Although Rah and Rab5 were colocated in some vesicles, the Rah-containing vesicles were generally present more peripherally in the cells, whereas the Rab5-containing vesicles occupied more central areas of the cells. These results suggest that, during macropinosome biogenesis, Rah acts at early stages and Rab5 functions at later stages and that Rah is replaced by Rab5 on macropinosomes.

The formation of membrane ruffles and macropinosomes are induced by the treatment of cells with growth factors or PMA (7–9, 11–13). Introduction of constitutively activated Ras or Rac1 or Tiam1, a GEF for Rac1, also results in prominent membrane ruffling and the formation of macropinosomes (13–15). This action of Ras is mediated by Rac1 because the dominant-negative Rac1(T17N) prevents this effect of Ras (13). Tiam1 is directly associated
with GTP-bound Ras and causes activation of Rac1 in a phosphatidylinositol 3-kinase (PI3K)-independent manner (54). Alternatively, PI3K, a target protein of Ras, may link Ras with Rac1 through its lipid product phosphatidylinositol 3,4,5-trisphosphate (PI(3,4,5)P₃). This lipid binds to and activates Vav and Sos, which serve as the GEFs for Rac1 (55, 56). Rac1 activated by these pathways, in turn, induces membrane ruffling by activating WAVE2 through the target protein IRSp53 (16–18). Induction of membrane ruffling and macropinocytosis by a short-term treatment of cells with PMA is mediated by Rac (13) or presumably by protein kinase Cε, which is activated by the PI3K product PI(3,4,5)P₃ (52). A presumable pathway for growth factor-induced membrane ruffling and macropinocytosis is summarized in Fig. 8.

We showed here that the expression of Rah(wt) and Rah(Q111L) facilitated the formation of macropinosomes over the control level. In contrast, the dominant-negative Rah(T66N) slightly retarded the macropinosome formation under the control level. When the cells were treated with PDGF or PMA, membrane ruffling and macropinosome formation were promoted. Expression of Rah(wt) in the PDGF- or PMA-treated cells further promoted the macropinosome formation. Expression of the dominant-negative Rah reduced the macropinosome formation, but the levels remained higher than the control level. On the other hand, the degree of membrane ruffling was not affected by any of these Rah proteins. These results indicate that Rah is concerned with macropinosome formation synergistically with growth factors (including serum growth factors in the growth medium) or PMA. Since Rah is not involved in membrane ruffling, however, Rah does not seem to constitute the signaling pathway activated by growth factors (Fig. 8).

We further showed that Rah-induced macropinosome formation was retarded to some degree by the dominant-negative H-Ras(S17N). On the other hand, Rac1(T17N) almost completely inhibited macropinosome formation, whereas the dominant-negative WAVE2(ΔV) suppressed it close to the control level. The only moderate retardation by H-Ras(S17N) might be ascribed to the presence in 10T1/2 cells of multiple endogenous Ras proteins (H-Ras, K-Ras, and N-Ras), which are structurally closely related but functionally distinctive (58, 59). Indeed, K-Ras generates membrane ruffles and macropinosomes more prominently than does
H-Ras probably because K-Ras activates Rac1 more efficiently than H-Ras (60). Thus, if GEFs specifically act on each member of Ras \textit{in vivo} and H-Ras(S17N) sequester a particular GEF, H-Ras(S17N) may not efficiently interfere with macropinosome formation. Although WAVE2 is activated by Rac1–IRSp53 to induce membrane ruffling and subsequent macropinocytosis, there are two other WAVE isoforms, WAVE1 and 3 (18, 20, 38). In addition, other Rac1 target proteins such as PAK1 may be implicated in the macropinocytosis pathway by inducing membrane ruffling (61, 62). These facts seem to be responsible for the incomplete inhibition of macropinosome formation by WAVE2(∆V).

Because macropinosomes are formed from membrane ruffles, macropinosome formation primarily requires membrane ruffling (4). Membrane ruffling induced by growth factors or PMA may result in spontaneous basal level macropinosome formation, which does not require the aid of Rah. Rah was colocalized with actin to the membrane ruffles and macropinosome membranes. Thus, the primary role of Rah seems to be the formation of macropinosomes by closing membrane ruffles through regulating actin reorganization. In this manner, Rah might be concerned with efficient macropinosome formation synergistically to the action of growth factors or PMA. This postulation is supported by the findings that Rah(T66N) is associated with membrane ruffles but not with macropinosomes and that both Rah and the actin coat disappear from macropinosomes as the vesicles migrate to the cell center. In this context, it should be noted that PI3K is not necessary for membrane ruffling but rather functions in the closure of membrane ruffles to form macropinosomes and phagosomes in macrophages (63). If this is also the case for fibroblasts, PI3K and PI(3,4,5)P_3 may participate in the activation of Rah (Fig. 8). The closure of membrane ruffles is accompanied by membrane fusion process. Some Rab family proteins including Rab5 are involved in vesicle membrane fusion (23, 24). Thus, it is of interest to examine whether Rah is required for the plasma membrane fusion at the ruffles. Identification of target proteins of Rah and their binding proteins may help to answer these questions.

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**FOOTNOTES**

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The nucleotide sequence reported in this paper has been submitted to the DDBJ/EMBL/GenBank with accession number AB082927.

1The abbreviations used are: PMA, phorbol 12-myristate 13-acetate; GEF, guanine nucleotide exchange factor; PDGF, platelet-derived growth factor; RT-PCR, reverse transcription-polymerase chain reaction; wt, wild-type; GST, glutathione S-transferase; DTT, dithiothreitol; DME medium, Dulbecco’s-modified Eagle’s medium; mAb, monoclonal antibody; HA, hemagglutinin; EGFP, enhanced green fluorescent protein; 10T1/2, C3H/10T1/2; RITC, rhodamine B isothiocyanate; GAP, GTPase-activating protein; PI3K, phosphatidylinositol 3-kinase; PI(3,4,5)P3, phosphatidylinositol 3,4,5-trisphosphate.
FIGURE LEGENDS

Fig. 1. **Amino acid sequence homology among Rah and other Rab family proteins.** (A) The amino acid sequence of mouse Rah and comparison of its sequence with those of several Rab family proteins. The N-terminal sequence of Rah upstream from Ser53, which is indicated by an arrow, is not included in the previously reported truncated clone (31). The first 5 nucleotides (AATTC) in the truncated cDNA clone in Morimoto et al. (31) seem to be derived from an EcoRI linker. The compared Rab family proteins are human Rab1 (accession number, M28209), Rab3A (M28210), Rab4 (M28211), Rab5 (M28215), Rab7 (X93499), and Rab9 (U44103). The amino acid sequences are aligned to search similarity by the method of Lipman and Pearson (64) and by eye. Amino acids at positions of >50% identity are shown in white on black. G1–G4, conserved core motifs for GTP/GDP-binding and GTPase activities; E, effector domain; asterisks, conserved C-terminal Cys residues. (B) Comparison of Rah and Rab36 sequences.

Fig. 2. **GTP-binding and GTPase activities of Rah and Rab5.** (A) *In vitro* GTP-binding/GTPase assay. GST-tagged wt, constitutively active mutants (CA), and dominant-negative mutants (DN) of Rah and Rab5 were loaded with [α-32P]GTP and incubated for 30 min at 37°C. Bound GTP/GDP were analyzed by thin-layer chromatography. The positions of GTP and GDP are indicated. (B) *In vivo* GTP-binding/GTPase assay. Myc-tagged wt and constitutively active mutants (CA) of Rah and Rab5 as well as the empty vector (control) were transiently transfected to COS-1 cells. The cells were incubated with 32Pi for 12 h to label the proteins. Each of these proteins was immunoprecipitated with the anti-Myc mAb and the bound GTP/GDP were analyzed by thin-layer chromatography.

Fig. 3. **Colocalization of Rah with actin to membrane ruffles and membranes of large vesicles adjacent to the ruffles.** Myc-tagged Rah(wt) (a–d), Rah(Q111L) (e–h), or
Rah(T66N) (i–l) was expressed in 10T1/2 cells. The cells were doubly stained with anti-Myc mAb Myc-1-9E10 and rhodamine–phalloidin. Shown are phase-contrast images (a, e, and i), Myc-1-9E10 staining locating Rah (b, f, and j), rhodamine–phalloidin staining locating actin filaments (c, g, and k), and their merged fluorescent images (d, h, and l). In the merged images, colocalization of Rah and actin filaments is seen in yellow. Arrowheads, chevrons, and arrows point to the membrane ruffles, vesicles neighboring the ruffles, and vesicles away from the ruffles, respectively. Scale bar, 20 µm.

Fig. 4. Partial coexistence of Rah and Rab5 in endosome membranes. (A) Localization of Rab5 in endosome membranes. Myc-tagged Rab5(wt) (a–c), Rab5(Q79L) (d–f), or Rab5(S34N) (g–i) was expressed in 10T1/2 cells. The cells were doubly stained with Myc-1-9E10 to locate Rab5 (a, d, and g) and rhodamine–phalloidin to locate actin filaments (b, e, and h). In the merged images (c, f, and i), colocalization of Rab5 and actin filaments is seen in yellow. Only some but not all of the Rab5-linked endosomes are associated with actin. Arrows point to the Rab5-linked endosomes partially colocalized with actin. (B) Distribution of Rah- and Rab5-containing vesicles. EGFP-tagged Rab5(Q79L) and Myc–Rah were coexpressed in 10T1/2 cells. Shown are EGFP fluorescence locating Rab5(Q79L) (a and d), Myc-1-9E10 staining locating Rah (b and e), and their merged images (c and f). Arrowheads, chevrons, white arrows, and black arrows point to the Rah-containing membrane ruffles, the vesicles exclusively containing Rah, those containing both Rah and Rab5, and those exclusively containing Rab5, respectively. Scale bar, 20 µm.

Fig. 5. Association of Rah with nascent macropinosomes but not with late endosomes or lysosomes. (A) Distribution of Rah and Rab5 compared with that of Lamp1 in late endosomes and lysosomes. EGFP-tagged Rah (a–c) or Rab5(Q79L) (d–f) was expressed in 10T1/2 cells. Shown are EGFP fluorescence (a and d), anti-Lamp1 staining (b and e), and their merged images (c and f). Arrows indicate late endosomes, where Rab5(Q79L) and
Lamp1 coexist. A chevron denotes putative lysosome, where Lamp1 but not Rab5(Q79L) exists. (B) Incorporation of rhodamine–dextran in Rah- or Rab5-associated endosomes. EGFP-tagged Rah (a–c) or Rab5(Q79L) (d–f) was expressed in 10T1/2 cells. RITC–dextran was added to the medium and incubated for 1 h. Shown are EGFP fluorescence (a and d), RITC–dextran incorporation (b and e), and their merged images (c and f). Arrows point to RITC–dextran-incorporating endosomes (macropinosomes). Scale bar, 20 µm.

Fig. 6. Association of Rah with macropinosome membranes formed from the membrane ruffles. EGFP-tagged Rah(wt) was expressed in 10T1/2 cells and EGFP fluorescence was recorded at several min intervals at 37°C. The numbers indicate min after starting the observation. Shown are selected figures of reverse color images. An arrow indicates a Rah-associated macropinosome formed from the membrane ruffle and migrating toward the cell center.

Fig. 7. Promotion of macropinosome formation by Rah. (A) Increase in the number of macropinosomes in Rah-expressing cells. Myc-tagged Rah(wt), Rah(Q111L), or Rah(T66N) was transfected to 10T1/2 cells. The number of actin-coated macropinosomes per cell was counted. The values are the means ± SD of triplicate experiments. (B) Elevation and reduction in the number of macropinosomes by the expression Rah(wt) and Rah(T66N), respectively, in PDGF- or PMA-treated cells. Myc–Rah(wt)- or Myc–Rah(T66N)-transfected 10T1/2 cells were treated with 5 U/ml PDGF or 0.1 µM PMA for 30 min. The number of actin-coated macropinosomes per cell was counted. The values are the means ± SD of triplicate experiments. (C) Influence of dominant-negative mutants of H-Ras, Rac1, and WAVE2 on Rah-promoted macropinosome formation. HA-tagged Rah(wt) was cotransfected with Myc–H-Ras(S17N), Myc–Rac1(T17N), or FLAG–WAVE2(ΔV) in 10T1/2 cells. The number of actin-coated macropinosomes per cell was counted. The values are the means ± SD of triplicate experiments. (D) Colocalization of Rah and WAVE2 to membrane ruffles and
macropinosomes. Myc–Rah(wt) and EGFP–WAVE2 were cotransfected to 10T1/2 cells. Shown are EGFP fluorescence locating WAVE2 (a), Myc1-9E10 staining locating Rah (b), and their merged image (c). An arrowhead and arrows point to the membrane ruffles and macropinosomes, respectively, containing both WAVE2 and Rah.

Fig. 8. A scheme of synergistic effect of Rah on the growth factor-induced macropinocytosis. Growth factor-induced membrane ruffling, which is mediated by Rac1–IRSp53–WAVE2, leads to a basal level macropinosome formation. Rah seems to be synergistically concerned with efficient macropinosome formation possibly by closing the membrane ruffles.
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Small GTPase Rah/Rab34 is associated with membrane ruffles and macropinosomes and promotes macropinosome formation

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