Expression and characterization of Rab38, a new member of the Rab small G protein family

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Abstract

Rab38 is a new member of the Rab small G protein family that regulates intracellular vesicle trafficking. Rab38 is expressed in melanocytes and it has been clarified that a point mutation in the postulated GTP-binding domain of Rab38 is the gene responsible for oculocutaneous albinism in chocolate mice. However, basic information regarding recombinant protein production, intracellular location, and tissue-specific expression pattern has not yet been reported. We produced recombinant Rab38 using a baculovirus/insect cell-protein expression system. A combination of Triton X-114 phase separation and nickel-affinity chromatography yielded exclusively prenylated Rab38 that bound [α-32P]GTP. The mRNA and the native protein were expressed in a tissue-specific manner, e.g., in the lung, skin, stomach, liver, and kidney. Freshly isolated rat alveolar type II cells were highly positive for the mRNA signal, but the signal was rapidly lost over time. Immunofluorescence staining demonstrated that expressed GST-tagged Rab38 was mainly colocalized with endoplasmic reticulum-resident protein and also partly with intermittent vesicles between the endoplasmic reticulum and the Golgi complex. These results indicate that Rab38 is expressed non-ubiquitously in specific tissues and regulates early vesicle transport relating to the endoplasmic reticulum, and hence suggest that Rab38 abnormality may cause multiple organ diseases as well as oculocutaneous albinism.

Keywords: alveolar type II cells; chocolate mice; endoplasmic reticulum; intracellular localization; Triton X-114 phase separation.

Introduction

Rab small G proteins are a group of monomeric intracellular proteins that regulate intracellular vesicle transport, including exocytosis, endocytosis, and transcytosis (Olkkonen et al., 1997; Takai et al., 2001). There are several regulator proteins for Rab proteins, including Rab GDP dissociation inhibitor (Rab GDI), Rab guanine nucleotide exchange protein (Rab GEP), and Rab GTPase activating protein (Rab GAP). Moreover, Rab proteins require effector proteins, e.g., Rabphilin-3 for Rab3A and Rabaptin-5 for Rab5 (Zerial and McBride, 2001). Information from the human genome sequence database and expressed sequence tags predict that the Rab GTPase protein family contains approximately 60 members. Thus, the Rab family is the largest family within the ras-oncogene small GTPase protein superfamily. However, not all Rab family members have been identified. Moreover, the intracellular localization and specific vesicular trafficking role have been determined for only a limited number of Rab proteins.

Rab proteins are prenylated at their carboxyl-termini, and some Rab proteins are also modified by palmitoylation. These post-translational modifications are essential for the biological activity of Rab proteins, because prenylated C-terminal domains are required to localize to specific cellular organelles. Rab proteins are found in both membrane-bound and cytosolic forms. They mediate intracellular vesicle transport between restricted intracellular compartments. Each Rab family member is strictly localized to defined intracellular organelles. Thus, the intracellular location of a Rab protein provides information that can be used to infer its function.

A single cell does not express all of the Rab family members. Many Rab proteins identified so far, such as Rab1, Rab2, Rab4, Rab5, Rab6–14, Rab18, Rab20, Rab22, Rab24, Rab28, and Rab30, are ubiquitously expressed in a variety of tissues and cells (Olkkonen et al., 1997; Zerial and McBride, 2001). However, the expression of some Rab proteins, including Rab3, Rab15, Rab17, Rab19, Rab21, Rab23, and Rab25–27, is highly regulated depending on the cell type (epithelial, neuronal, endocrine, or exocrine) and specific phenotype (differentiation or polarization) (Olkkonen et al., 1997; Zerial and McBride, 2001). The types of cells and tissues in which a Rab protein is expressed provide information regarding the protein’s function.

Rab38 is a novel member of the Rab family. The cDNA for Rab38 was originally cloned from a rat lung cDNA library (GenBank accession no. M94043), and was later cloned from a human melanoma cDNA library (Jager et al., 2000). The amino acid sequence deduced from Rab38 cDNA predicts that the molecular weight of this protein is 24 kDa. We previously showed that Rab38 was specifically expressed in alveolar type II cells and Clara
cells in rat lung tissue (Osanai et al., 2001a). In addition, a point mutation in the conserved consensus sequence of the GTP/GDP-binding domain of Rab38 has been reported to cause ocu-lod, mouse albinism in C57BL/6J mice (Loftus et al., 2002). Although three previous reports have described Rab38, these reports did not contain systematic information. The goal of the present study was: (i) to determine the organs that express Rab38; (ii) to purify and characterize recombinant Rab38 protein; and (iii) to determine the intracellular localization of Rab38.

Results

Purified recombinant Rab38 is exclusively isoprenylated

Recombinant Rab38 was expressed in Sf9 cells that were transfected with recombinant baculovirus carrying 6-histidine-tagged Rab38 cDNA. Figure 1A shows an SDS-PAGE gel that contains samples of proteins from different purification steps. The post-nuclear supernatant from infected Sf9 cells that were lysed with Triton X-114 was used as the starting sample. The purification strategy combined Triton X-114 phase partitioning and nickel-affinity chromatography. After elution with an imidazole gradient, recombinant Rab38 appeared as a 27-kDa protein in SDS-PAGE. After the recombinant protein was separated by SDS-PAGE and transferred to a nitrocellulose membrane, the immobilized protein bound $\alpha-[32P]GTP$ in a dose-dependent manner, indicating that this protein has specific GTP-binding activity (Figure 1B). When transfected Sf9 cells were radiolabeled with $[^{35}S]$-methionine and $[^{35}S]$-cysteine and subjected to Triton X-114 phase partitioning, newly synthesized Rab38, which was immunoprecipitated with anti-rat Rab38 antibody, was recovered in both the aqueous and detergent phases (Figure 2A, lanes 1–3). When transfected Sf9 cells were treated with mevinolin, a 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase inhibitor that blocks de novo cholesterol synthesis to inhibit isoprenoid modification, newly synthesized Rab38 was exclusively recovered into the aqueous phase and not into the detergent phase (Figure 2A, lanes 4–6). In contrast, when the cells were radiolabeled with $[^{3}H]$-mevalonate in the presence of mevinolin, radiolabeled Rab38 was extensively recovered into the detergent phase, indicating that all isoprenylated Rab38 is recovered in the Triton X-114 detergent phase (Figure 2B).

Rab38 is not expressed ubiquitously

The levels of Rab38 mRNA as determined by quantitative RT-PCR varied in different rat organs (Figure 3A). The highest Rab38 mRNA level was detected in the lung, followed by the skin, stomach, kidney, and liver. Freshly isolated rat alveolar type II cells had a higher signal than total lung tissue, but type II cells quickly lost the mRNA signal when cultured in plastic dishes (Figure 3B). Type II cells that were cultured overnight in plastic dishes almost completely lost the mRNA signal. Alveolar macrophages, A549 cells, COSS-7 cells, and 293T cells did not express Rab38 mRNA. Western blot analysis confirmed the organ-specific expression pattern determined by quantitative RT-PCR (Figure 4). The intensity of immunoreactive Rab38 in rat organ lysates was highest in the lung, followed by the skin, liver, kidney, and stomach. Perfused rat livers and kidneys were rapidly frozen and cut into thin slices that were immunostained with an affinity-purified rabbit anti-Rab38 antibody (Figure 5). In the liver, hepatocytes were extensively stained with the Rab38 antibody, but Glisson capsules (i.e., the interlobular hepatic artery and vein, and interlobular bile ducts) were not stained. In the kidney, the proximal renal tubular epithelial cells, but not the glomerulus, were stained with the Rab38 antibody.
The cytoplasm of intact COS cells transfected with cytosolic proteins while keeping the cell structures intact. The cytosolic protein that reacted with both rabbit anti-GST antibody but not with rabbit anti-Rab38 antibody (Figure 6, lane 2). COS cells were not stained by the anti-GST antibody. This suggests that the GST produced by transfected COS cells was a cytosolic protein that spilled out of the permeabilized cells completely. Figure 7A shows the intracellular localization of various organelle marker proteins in A549 cells. A549 cells appeared to provide better information regarding intracellular organelle distribution than the COS cells. Figure 7B shows the co-localization (c,f) of recombinant GST-tagged Rab38 (a,d) and KDEL (b,e), an endoplasmic reticulum marker protein, in A549 cells (a–c) and COS cells (d–f). Among the cellular organelles examined, GST-tagged Rab38 appeared to best co-localize with KDEL in both A549 and COS cells. This result was also confirmed in non-transfected B16 murine melanoma cells (m–o). The native Rab38 was best co-localized with KDEL. GST-tagged Rab38 also appeared to partially co-localize with βCOP (g–i) and ERGIC (data not shown, but the result was similar to that of βCOP) and GM130 (j–l).

Rat liver was homogenized and fractionated by differential and sucrose density-gradient centrifugation. The fractionated samples were used for Western blotting (Figure 8). The same nitrocellulose membrane was sequentially probed with several types of first antibodies to compare the distribution pattern of Rab38 and organelle marker proteins among separated liver organelles. Rab38 was more enriched in the pellet centrifuged at 100 000 g than that at 15 000 g. However, Rab38 showed a broad distribution pattern, including in the cytosol fraction. Among several organelle marker proteins tested, the distribution pattern of KDEL/GRP78 appeared to be similar to that of Rab38, except for the cytosol fraction. The anti-KDEL antibody reacted with Grp78 (BiP) and Grp94, the ER marker proteins.

### Discussion

We used the baculovirus/insect cell protein expression system to produce recombinant Rab38, in which a fusion peptide containing a 6-histidine site was added to the N-terminus. The infected Sf9 cells were lysed on ice with a 1% solution of Triton X-114, a non-ionic detergent. Triton X-114 is homogenous at 0–4°C but separates into an aqueous phase and a detergent phase at temperatures greater than 20°C after low-speed centrifugation. Hydrophilic proteins are found in the aqueous phase, and amphiphilic or hydrophobic proteins are recovered in the detergent phase (Bordier, 1981). We loaded the cell lysate components recovered in the detergent phase onto a nickel-affinity column. Thus, this lysate presumably contained amphiphilic or hydrophobic proteins. Our goal was to purify only Rab38 that was prenylated, because non-prenylated Rab proteins are not biologically active (Lapetina and Reep, 1987). Membrane attachment depends on the addition of geranylgeranyl, a C-20 isoprenyl lipid, to one or two cysteines at the carboxyl-terminus of Rab proteins (Araki et al., 1991). The detergent-soluble sample was already considerably purified by the removal of pCEFL-GST plasmid was only homogeneously stained positive by anti-GST antibody (data not shown). However, after the transfected cells were permeabilized, they were not stained by the anti-GST antibody. This suggests that the GST produced by transfected COS cells was a cytosolic protein that spilled out of the permeabilized cells completely.
Figure 3  Quantitative competitive RT-PCR of Rab38 in total RNA extracted from (A) rat organs and (B) alveolar type II cells and other cells. After treatment with DNase I, first-strand DNA was synthesized with oligo(dT) primers using reverse transcriptase. In the presence of 10-fold serial dilutions of PCR competitor (i.e., known concentrations of site-deleted Rab38-cDNA), the first PCR cycle was carried out. Aliquots of the PCR product were electrophoresed and stained. The two consecutive 10-fold dilutions, between which equal amounts of site-deleted cDNA and the first PCR product were expected to be present, were determined. Then, two-fold serial dilutions of the PCR competitor (between the two selected 10-fold dilutions) were added to the second PCR reaction mixture. After the second PCR cycle, the concentration of Rab38 mRNA was determined from the known concentration of the site-deleted Rab38-cDNA competitor that resulted in approximately equal intensities of the two PCR products with different molecular sizes. Data were normalized against those of GAPDH. (A) Lane 1, brain; lane 2, thymus; lane 3, lung; lane 4, heart; lane 5, liver; lane 6, stomach; lane 7, pancreas; lane 8, kidney; lane 9, large intestine; lane 10, small intestine; lane 11, skeletal muscle; and lane 12, skin. (B) Lane 1, lung; lane 2, freshly isolated rat alveolar type II cells; lane 3, type II cells cultured overnight; lane 4, type II cells cultured for 3 days; lane 5, type II cells cultured for 7 days; lane 6, rat alveolar macrophages; lane 7, A549 cells; lane 8, COS-7 cells; and lane 9, 293T cells. Note that isolated alveolar type II cells completely lose the mRNA signal after overnight culture in plastic dishes.

Figure 4  Western blot of native Rab38 protein expressed in rat organs and in B16 murine melanoma cells. Total proteins were extracted from extensively perfused and homogenized organs with 1% Triton X-100 with protease inhibitors. Equal amounts of protein (100 µg), except for B16 cells (50 µg), were subjected to SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was immunoblotted with a rabbit anti-rat Rab38 antibody followed by a horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin antibody. Color development was carried out in the presence of 0.03% H2O2 and 1 mg/ml of diaminobenzidine within 3 min. Lanes 1 and 10, prestained protein standards; lane 2, lung, lane 3, heart; lane 4, liver; lane 5, kidney; lane 6, stomach; lane 7, skeletal muscle; lane 8, skin; and lane 9, B16 cells.

Figure 5  Immunohistochemical stain of rat liver and kidney. Organs were extensively perfused with phosphate-buffered saline (PBS) from the ascending aorta through the left atrium. The organs were excised, cut, embedded in OCT compound, rapidly frozen in liquid nitrogen and stored at -80°C. Thin slices (5–7 µm thick) were fixed with 4% paraformaldehyde/PBS for 10 min and then 100% acetone for 30 s. Rabbit anti-rat Rab38 antibody (1.6 µg/ml) (A,C) or normal rabbit IgG (1.6 µg/ml) (B,D) were added, followed by biotin-labeled goat anti-rabbit IgG antibody. Upper (A,B): liver; lower (C,D): kidney. Magnification ×50.
Rab38 is selectively expressed in specific cells of specific organs and is probably associated with a specialized function of these cells.

Each Rab protein or its counterpart in yeast is found at a particular stage of the membrane transport pathway (Olkkonen et al., 1997; Takai et al., 2001; Zerial and McBride, 2001). For example, SEC4 is found at the site of cell surface expansion, associated with secretory vesicles and the plasma membrane. YPT1 is associated with the Golgi apparatus and the endoplasmic reticulum-Golgi apparatus carrier vesicles. Rab5 is associated with early endosomes and the plasma membrane. Proteins of the SEC4/Ypt/Rab family undergo a cycle of localization as they fulfill their functions. There are three pools of SEC4: a small soluble pool, a pool associated with the cytoplasmic surface of secretory vesicles, and a pool associated with the inner surface of the plasma membrane. Newly synthesized SEC4 rapidly associates with secretory vesicles that go on to fuse with the plasma membrane. The plasma membrane-bound pool can recycle into a new round of vesicles as they accumulate in a secretory mutant blocked in exocytosis. This recycling pathway may utilize a soluble intermediate, Rab3A, which is predominantly associated with synaptic vesicles, has also been shown to undergo redistribution following exocytic fusion (Fischer von Mollard et al., 1991). Thus, it is a characteristic feature of Rab proteins that their intracellular localization is strictly defined to a particular recycling pathway (i.e., transporting vesicle, target membrane, and cytosol). COS cells transfected with pCEFL-GST or pCEFL-GST-Rab38 produced substantial amounts of GST or GST-tagged Rab38, respectively (Fig-
ure 6). Confocal laser microscopic analysis of immunofluorescence cytochemistry performed on B16 cells, transfected A549 cells, and transfected COS cells showed that the distribution pattern of Rab38 most closely matched that of KDEL (the tetrapeptide located at the C-terminal of luminal proteins in the ER, a retrieval motif for these proteins from the ER along the secretory pathway) (Figure 7B), suggesting that the predominant location of Rab38 is the endoplasmic reticulum. Rab38 also partially co-localized with ERGIC, βCOP, and the Golgi apparatus. However, care must be taken in assuming partial co-localization of Rab protein with organelles other than the endoplasmic reticulum, because the broad distribution pattern of Rab38 may overlap with these organelles. Nonetheless, it is likely that Rab38 partially co-localizes with the vesicles between the endoplasmic reticulum and the Golgi apparatus, because vesicle components transported from the endoplasmic reticulum to the Golgi apparatus should be recycled back to the endoplasmic reticulum (Mellman and Warren, 2000). In COS cells, co-localization of Rab38 and KDEL appeared to be condensed in the Golgi apparatus (Figure 7B, d–f).

The close relationship of Rab38 with the endoplasmic reticulum is also supported by the result from rat liver homogenate. In separated fractions from rat liver homogenate, Rab38 was more enriched in the 100 000 g pellet than in the 15 000 g pellet. Although Rab38 showed a broad distribution pattern, the distribution pattern of KDEL/GRP78 among those of several organelle marker proteins appeared to be similar to that of Rab38, except for the cytosol fraction. The broad distribution pattern of Rab38 is likely because Rab38 is not a membrane-spanning protein or an encapsulated protein in the vesicle, but is a protein that binds to and dissociates from vesicles, depending on a GTP/GDP switch. The broad distribution pattern may reflect the heterogeneous density of membrane components to which Rab38 binds.

It was recently clarified that a naturally occurring mutation of ocucutaneous albinism in chocolate (cht) mice was caused by a genetic disorder of rab38 (Loftus et al., 2002). Genomic DNA analysis revealed a G146T mutation in either Tyr or cht/cht mice caused glycine to be replaced with valine in the conserved GTP-binding domain of Rab38. Rab38chtccht mice exhibit a brown coat and eyes similar in color to those of the brown mice with a mutation in tyrosinase-related protein (Tyrp1), a mouse model for human ocucutaneous albinism type 3. Various types of ocucutaneous albinism are associated with reduced pigmentation in the skin, hair, and eyes that results from mutations in genes involved in melanin synthesis. Oculocutaneous albinism types 1 and 3 are endoplasmic reticulum retention diseases in which a mutation in tyrosinase or Tyrp1 can affect the processing of both mutant and wild-type proteins (Toyofuku et al., 2001). A mutation in either Tyr or Tyrp1 increases the time of association of tyrosinase and Tyrp1 with calnexin and Bip, which results in the retention of these mutant products in the endoplasmic reticulum and markedly slows their transport to melanosomes. The present study indicated that Rab38 was mostly localized to the endoplasmic reticulum. It is likely that Rab38 participates in the transport of one or more melanogenic substances at the level of the endoplasmic reticulum to the Golgi apparatus.

More recently, the rat Ruby (R) locus has been attributed to Rab38 (Oiso et al., 2004). The phenotype of the R mutation in rats shows ocucutaneous albinism, plate-
let storage pool defects, and possibly pulmonary diseases. Analysis of genomic DNA of these rats (Fawn-hooded rats and Tester Moriyama rats) showed Met116 substitution in Rab38, which results in a protein-null defect, abolishing translation from the mutant allele. Thus, Fawn-hooded and Tester Moriyama rats are considered to be rat models of Hermansky-Pudlak syndrome. It is not clear why chocolate mutant mice and Ruby mutant rats show important phenotypic differences, i.e., bleeding diathesis and several other organ diseases. Chocolate mice were reported to show only oculocutaneous albinism and no bleeding disorder (Lofthus et al., 2002).

Although the precise role of Rab38 remains to be elucidated, we found that Rab38 is selectively expressed in several specific tissues, including alveolar type II cells and melanocytes, and appears to participate in endoplasmic reticulum-related transport. These results predict that Rab38 abnormality may cause multiple organ diseases, as well as oculocutaneous albinism.

Materials and methods

Chemicals and reagents

Common chemicals and reagents were purchased from Sigma (St. Louis, USA) or Wako Chemicals (Osaka, Japan). Restriction enzymes and DNA molecular weight markers were purchased from WAKO Chemicals. The following antibodies were used for immunostaining and immunoblot: anti-KDEL, Stressgen No. SPA-827 (Victoria, BC, Canada); jICOP, Sigma no. G6160 (Sigma); SEC23 no. sc-12107 and Lamp-1 no. sc-8098, Santa Cruz (Santa Cruz, USA); GM130 no. G65120, TGN38 no. T69020, and EEA1 no. E41120, Transduction Laboratories (Lexington, USA); Alexa488-conjugated goat anti-rabbit IgG no. A-11008, Alexa594-conjugated goat anti-mouse IgG no. A-10112, and Alexa647-conjugated donkey anti-rabbit IgG no. A-21206, Molecular Probes (Eugene, USA); and Cy3.5-conjugated donkey anti-goat IgG, Rockland no. 605-712-125 (Gilbertsville, USA). Mouse anti-human ERGIC-53 monoclonal antibody was a kind gift from Dr. Hans-Peter Hauri (Department of Pharmacology, Basel University, Switzerland).

Cells

A549 cells, COS-7 cells, and B16 cells were cultured in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Cells were used within 30 passages. B16 cells were obtained from the Cell Resource Center for Biomedical Research, Tohoku University, Japan. Alveolar type II cells were isolated from specific-pathogen-free adult male Sprague-Dawley rats (Sankyo Labo Service, Tokyo, Japan) by porcine pancreatic elastase (Worthington, Freehold, USA) digestion and metrizamide (Sigma) density-gradient centrifugation, according to the method described by Debs et al. (1980). Alveolar macrophages were isolated by bronchoalveolar lavage. In SPF rats, greater than 98% of the lavaged cells were macrophages and these cells were used without further purification. Spodoptera frugiperda cells (Sf9 cells) (Invitrogen, Carlsbad, USA) were cultured in TNM-FH medium (Gibco-BRL, Gaithersburg, USA) supplemented with 10% FBS in 25-cm plastic culture dishes.

Protein production and purification

The original Rab38 cDNA clone was constructed in a pET-3 vector. The Rab38 cDNA was amplified by PCR using specific primers (Osanai et al., 2001a). The PCR product was digested with BamHI and HindIII, and inserted into the pBlueBacHis-2A vector (Invitrogen). DNA sequencing of the recombinant vector was carried out with the ABI BigDye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Foster City, USA). Cationic liposome (InsectinPlus, Invitrogen) was used to co-transfect the recombinant plasmid and lethal-depleted baculovirus (Bac-N-Blue DNA, Invitrogen) into Sf9 cells according to the manufacturer’s instructions. The presence of recombinant virus that contained Rab38 was verified by PCR. Virus titers were determined by plaque assays. Cells that were 80–90% confluent in 25-cm plastic dishes were infected with the recombinant virus at a multiplicity of infection (MOI) of 10. The frozen Sf9 cells were rapidly thawed and suspended in lysis buffer (1% TritonX-114, 50 mM HEPES, pH 7.4, 150 mM NaCl, 1.5 mM EGTA, and 10% glycerol) containing protease inhibitors (1 mM PMSF, 10 μg/ml aprotinin, and 10 μg/ml leupeptin). The post-nuclear supernatant was extracted using Triton X-114 phase partitioning (Bordier, 1981). The supernatant with 1% Triton X-114 was layered onto a 6% sucrose cushion (6% sucrose, 50 mM HEPES, pH 7.4, 150 mM NaCl, and 0.06% Triton X-114). It was then incubated at 30°C in a water bath for 30 min and centrifuged at a low speed for 10 min in a swinging bucket rotor. The upper layer was recovered, adjusted to 0.5% Triton X-114, incubated at 30°C for 30 min, layered onto the previous sucrose cushion, and centrifuged for another 10 min. The upper layer was recovered and referred to as the aqueous phase. The detergent pellet in the bottom of the tube was dissolved in phosphate-buffered saline (PBS) and is referred to as the detergent phase. The detergent phase was loaded on a Ni2+–charged affinity column (Probond, Invitrogen) under native conditions, according to the manufacturer’s instructions (Janknecht et al., 1991). Imidazole gradients were used to elute the column. The purified fraction was monitored with SDS-PAGE and subsequent Coomassie Brilliant Blue staining.

GTP-binding assay

Purified recombinant Rab38 was loaded on SDS-PAGE under reducing conditions and electrophoretically transferred to a nitrocellulose membrane. [α-32P]-GTP binding to the protein immobilized on the membrane was examined as previously described (Lapetina et al., 1987). The membrane was incubated for 1.5 h at 25°C in 100 ml of 50 mM Tris-HCl (pH 7.5) containing 5 mM MgCl2, 0.1% Triton X-100, and 0.1% BSA. Then the membrane was incubated with 60 nm [α-32P]-GTP (Amersham, Piscataway, USA, specific activity 3000 Ci/mmole) in 10 ml of the Tris-HCl buffer for 1.5 h at 25°C. The membrane was washed six times with 100 ml of the Tris-HCl buffer for 15 min at 25°C. The membrane was then dried and autoradiographed.

Radiolabeling of cells

Sf9 cells (1×10⁶ cells) were infected with the recombinant baculovirus at a MOI of 10 and cultured for 1 day. Then, the cells were radiolabeled with 100 μCi/ml of [35S]-methionine and [35S]-cysteine in methionine-deficient TNM-FH media (Gibco-BRL) for 3 days, with or without 5 μg/ml of mevinolin, or radiolabeled with 5 μCi/ml of [3H]-mevalonate in the presence of mevinolin (Lowe et al., 1990). Mevinolin was added 3 h before adding the radiolabeled. The cells were cultured in TNM-FH medium supplemented with 10% FBS previously dialyzed against PBS. The cells were lysed with 1% Triton X-114 and divided into two samples. One sample was used as the total cell lysate and the other was phase-separated using Triton X-114. Both samples were
immunoprecipitated with an rabbit anti-rat Rab38 antibody (5 μg/ml; Osanai et al., 2001a) and were subjected to SDS-PAGE under reducing conditions, followed by autoradiography.

### Quantitative competitive PCR

Total RNA was extracted from perfused rat organs, alveolar type II cells, alveolar macrophages, and cell lines using an RNA isolation kit (Trizol, Gibco-BRL). DNase I (an RNase-free DNase; Promega, Madison, USA) was added to the total RNA sample and incubated at 37°C for 15 min. First-strand DNA was synthesized with oligo(dT) primers using a reverse transcriptase (Superscript RT II RT, Gibco-BRL). In the presence of 10-fold serial dilutions of competitor cDNA (known concentrations of site-deleted Rab38 cDNA), the first-strand DNA template, primers (Quantitative Competitive PCR Human Rab38 no. RAB-7001, Maxim Biotech, Inc, San Francisco, USA), Taq DNA polymerase (Takara Ex Taq, Takara, Tokyo, Japan), and other PCR reaction components were added. PCR was started by the hot start method at 94°C and was run for a total of 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 90 s. Aliquots of the PCR product were electrophoresed on a 2% agarose gel and stained with ethidium bromide. We then determined the two consecutive 10-fold dilutions of competitor cDNA between which equal amounts of the competitor cDNA and the first RT product appeared to be present. Then the PCR reaction was carried out using two-fold serial dilutions of the competitor cDNA that were between the two selected 10-fold dilutions. The concentration of Rab38 mRNA was then determined from the known concentration of competitor cDNA that resulted in two PCR products with different molecular sizes and approximately equal intensities. Data were normalized against those for GAPDH (Quantitative Competitive PCR Rat GAPDH Gene no. GAP-7201, Maxim Biotech Inc).

### Western blotting

Total protein was extracted from extensively perfused rat organs, isolated alveolar type II cells, alveolar macrophages, and several cell lines using lysis buffer with protease inhibitors. Protein concentrations were determined by deoxycholate-trichloroacetic acid precipitation and a BCA micro-protein assay kit (Pierce, Rockford, USA). SDS-PAGE was carried out under reducing conditions with 1-mm-thick, 8–16% Tris-glycine gradient, pre-cast minigels (Novex, San Diego, USA). After electrophoresis, the proteins separated in the gel were electrophoretically transferred to a nitrocellulose membrane. The membranes were reacted with 0.8 μg/ml of an affinity-purified rabbit anti-rat Rab38 polyclonal antibody or a 1:1000 dilution of rabbit anti-GST polyclonal antibody (Sigma G7781) in 3% skim milk, 1% Triton X-100, and PBS overnight at 4°C. The membranes were then reacted with a horseradish peroxidase-conjugated anti-rabbit IgG antibody (Bio-Rad 170-6615, Hercules, USA) at a 1:1000 dilution and captured affinity-purified donkey anti-goat IgG antibody (1:200 dilution) or Cy3.5-conjugated affinity-purified donkey anti-goat IgG antibody (1:200 dilution) were used. For B16 murine melanoma cells, rabbit anti-Rab38 antibody instead of the anti-GST antibody and goat anti-KDEL antibody were used as the primary antibody.

### Immunohistochemistry

Excised rat liver and kidney were embedded in OCT compound (Sakura Finetechnical, Tokyo, Japan), frozen rapidly with liquid nitrogen, and stored at -80°C until use. Organ slices 5–7 μm thick were prepared with a cryostat and fixed with 4% paraformaldehyde for 10 min, followed by 100% acetone for 30 s. The anti-Rab38 antibody was layered onto the fixed organ slices at a concentration of 1.6 μg/ml. Control staining was carried out using the same concentration of non-immune rabbit IgG. Immunostaining was performed based on the ABC method (Histofine SAB-PO Kit; Nichirei, Tokyo, Japan). Color development was carried out for 3 min in the presence of 1 mg/ml diaminobenzidine and 0.03% H2O2.

### Transfection of mammalian cells

A restriction enzyme (BamHI and EcoRI)-digested DNA fragment that contained Rab38 from the pBlueBacHis-2A vector was inserted into pcDNA3-GST (Zohar et al., 1998). COS or A549 cells were seeded in 35-mm plastic dishes or chamber slides (Lab-Tek 177437; Nalgen Nunc, Naperville, USA) the day before transfection to achieve 50–80% confluency on the day of transfection. COS cells were transfected with SuperFect transfection reagent (Qiagen, Valencia, USA). The ratio of plasmid DNA to transfection reagent (activated dendrimer) was 1:5, so that 2 μg of DNA and 10 μl of SuperFect reagent were used for cells plated in 35-mm dishes. After 24–48 h of culture, the transfected COS cells were used for Western blots or immunostaining. A549 cells were transfected using the lipofection method (GenePORTER 2; GTS Inc, San Diego, USA). A 2-μg sample of plasmid DNA (in 1.6 μl of TE) was diluted with 48.4 μl of DNA diluent B and mixed with 7 μl of Geneporter 2 reagent that was previously supplemented with 43 μl of DMEM. The mixture was then added to a slide chamber well. After 4 h, 100 μl of DMEM with 20% FBS was added. The following day, the medium was replaced with fresh DMEM containing 10% FBS. After 24–48 h, the transfected A549 cells were used for immunostaining.

### Double-fluorescence immunostaining and confocal laser microscopy

B16 cells, transfected A549 cells or COS cells cultured on chamber slides were fixed with 80% methanol/20% acetone at -20°C for 15 min, or 4% paraformaldehyde/PBS for 10 min followed by 100% acetone for 30 s. The slides were reacted for 1 h with a rabbit anti-GST polyclonal antibody (1:1000 dilution), and a mouse monoclonal antibody (KDEL at 1:100, ERGIC at 1:100, GM130 at 1:100, TGN38 at 1:100, or EEA1 at 1:100), or affinity-purified goat polyclonal antibody (Lamp-1 at 1:100 or SEC23 at 1:100). The slides were reacted for 30 min with secondary antibodies Alexa 488-labeled goat anti-rabbit IgG antibody (or Alexa 488-labeled donkey anti-rabbit IgG antibody) (1:400 dilution) and Alexa 594-labeled goat anti-mouse IgG antibody (or Alexa 594-labeled donkey anti-goat IgG antibody) (1:400 dilution). For Lamp-1 and SEC23, Alexa 488-conjugated affinity-purified donkey anti-rabbit IgG antibody (1:100 dilution) and Cy3.5-conjugated affinity-purified donkey anti-goat IgG antibody (1:200 dilution) were used. For B16 murine melanoma cells, rabbit anti-Rab38 antibody instead of the anti-GST antibody and goat anti-KDEL antibody were used as the primary antibody. Confocal microscopic images were obtained using a computer-interfaced, laser-scanning microscope (Leica TCS-4D). Immunolabeled slides (three–five representative fields per slide chamber) were optically sectioned through the cell monolayer to obtain the appropriate focal depth. The representative images that were chosen contained the nucleus and relevant organelles of interest (endoplasmic reticulum, Golgi complex, endoplasmic reticulum-Golgi complex intermittent vesicles, trans-Golgi network, endosomes, or lysosomes). Simultaneous wavelength scanning allowed superimposition of fluorescent labeling with Alexa 488 and Alexa 594 (or Cy3.5) fluorophores. Confocal images were obtained using the following parameters: voltage 450–550 and offset -1 for Alexa 488-labeled secondary antibody, and voltage 550–650, offset -1 for Alexa 594-labeled secondary antibody. The laser power was fixed at approximately 60% for image acquisition. The pinhole setting, which was identical for both Alexa 488 and Alexa 594 images due to simultaneous scanning, was fixed to 100. Image sizes (zoom) were X:
Fractionation of rat liver homogenate

Male Sprague-Dawley rats were injected intraperitoneally with 75 mg/100 g weight of non-ionic detergent Triton WR 1339 (Tyloxapol; Ruge Chemical, Irvington, USA) 3 days before the experiment, and the rats were starved overnight before the experiment. The liver was excised and extensively perfused with PBS, then homogenized with a Potter-Elvehjem-type homogenizer in 0.25 M sucrose in 10 mM Tris, pH 7.5. The homogenate was centrifuged at 600 g for 10 min. The post-nuclear supernatant was centrifuged at 15 000 g for 5 min. The supernatant was then centrifuged at 100 000 g for 60 min. The 15 000 g pellet was resuspended and layered on a density gradient in a centrifuge tube (density from bottom to top: 1.25, 1.22, 1.19, 1.15, 1.11, and 1.09). The gradient was centrifuged at 100 000 × g for 3 h. The visible bands were separately recovered. Each separated fraction was solubilized with 1% Triton X-100 with proteases. The protein concentration was determined with a commercial kit (Pierce). The separated fraction was solubilized with 1% Triton X-100 with proteases. The protein concentration was determined with a commercial kit (Pierce) after deoxycholate-trichloroacetic acid precipitation. Equal amount of protein (50 μg) were subjected to SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was immunoblotted with a rabbit anti-rat Rab38 polyclonal antibody or anti-organellar marker protein antibody followed by a horseradish peroxidase-conjugated goat anti-rabbit (or goat anti-mouse) immunoglobulin antibody. The chemiluminescent detection assay was carried out using a commercial kit (SuperSignal West Pico Chemiluminescent Substrate; Pierce). The same nitrocellulose membrane was used for blotting of a series of first antibodies after stripping off the previous antibody (Restore™ Western Blot Stripping Buffer; Pierce).

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References


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