The function of Rab24 is currently unknown, but other members of the Rab GTPase family are known to participate in various protein trafficking pathways. Rab proteins are thought to cycle on and off vesicle membranes in conjunction with changes in their guanine nucleotide state. The present studies indicate that Rab24 possesses several unusual characteristics that distinguish it from other Rab proteins. 1) Based on \[^{32}P\]orthophosphate labeling of protein-bound nucleotide, Rab24 exists predominantly in the GTP state when expressed in cultured cells. The low GTPase activity is related to the presence of serine instead of glutamine at the position cognate to Ras Gln-61. 2) Posttranslational geranylgeranylation of Rab24, determined by metabolic labeling or detergent partitioning assays, is inefficient when compared with other Rabs ending with the common CXC and CC carboxyl-terminal motifs. This is partly due to the presence of two histidines distal to the target cysteines, but also involves other unidentified features. 3) Most of the Rab24 in the cytoplasmic compartment of cultured cells is not associated with Rab GDP dissociation inhibitors. These findings indicate that, if Rab24 functions in vesicular transport processes, it may operate through a novel mechanism that does not depend on GTP hydrolysis or GDP dissociation inhibitor-mediated recycling.

The Rab proteins comprise the largest subgroup in the superfamily of Ras-related GTPases, with the current number of unique gene products exceeding 40. It is now well established that different Rab proteins are localized in discrete subcellular compartments in mammalian cells (1–3). In a few cases, extensive studies utilizing in vitro reconstitution assays or in vivo expression of dominant-negative mutants have demonstrated that specific Rab proteins function as molecular switches in distinct segments of the exocytic or endocytic vesicular transport pathways (3, 4). For example, Rab1A and Rab1B mediate constitutive anterograde protein trafficking from the endoplasmic reticulum to the cis-Golgi compartment (5, 6), whereas Rab5A directs early steps in endocytosis (7, 8). Although the aforementioned Rab proteins are expressed ubiquitously, others such as Rab3A, Rab3B, and Rab3D show restricted tissue distributions and appear to play specialized roles in regulated secretion or protein sorting in nerve terminals or endocrine cells (9–12).

The association of nascent Rab proteins with cell membranes is mediated by hydrophobic geranylgeranyl moieties, which are linked to COOH-terminal cysteine residues through the action of a multimeric enzyme complex, Rab-geranylgeranyltransferase (13). In addition, unique sequences in the hypervariable COOH-terminal region of each Rab protein are important for their specific subcellular targeting (14). The molecular details of Rab interaction with the vesicular transport machinery are still poorly understood. However, the most widely accepted models (3, 4, 15, 16) postulate that a specific Rab protein initially associates with budding vesicles of a specific donor membrane compartment, where it is activated by exchange of GDP for GTP. In the activated GTP state, the Rab protein promotes the interaction of specific components of the v-SNARE-t-SNARE complex in a manner that facilitates docking of donor vesicles with the correct acceptor compartment. Finally, in concert with vesicle fusion, the Rab protein reverts to the GDP state, either through its intrinsic GTPase activity or the action of a specific Rab GTPase-activating protein. Once in the GDP state, the Rab protein becomes a substrate for one of several Rab guanine nucleotide dissociation inhibitors (GDIs), which can extract geranylgeranylated Rabs from membranes and hold them in a cytosolic Rab-GDI complex until they are recruited into a new round of vesicular transport (17).

Despite the progress summarized above, the specific cellular functions of the majority of Rab proteins are currently unknown. Among these is Rab24, which was cloned from a mouse brain cDNA library by Olkkonen et al. (18). Although this protein clearly falls into the Rab subgroup, based on sequence conservation with other members of the Rab family, it contains some unusual structural features that attracted our attention. Herein we report that, unlike other Rab proteins, Rab24 exists predominantly in the GTP state and does not appear to form a stable complex with GDI in cultured cells. In addition, Rab24 is not efficiently geranylgeranylated when compared with other Rab proteins in cell-free systems or intact cells. In light of the importance of GTP hydrolysis, GDI-mediated recycling, and prenylation for the functioning of Rab proteins in vesicular transport, the atypical properties of Rab24 suggest that it may play a novel physiological role.

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‡ The abbreviations used are: GDI, GDP dissociation inhibitor; REP, Rab escort protein; FCR, polymerase chain reaction; HEB, human embryonal kidney; GGPP, geranylgeranyl pyrophosphate; PAG, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; PBS, phosphate-buffered saline; DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; MVA, mevalonate; FITC, fluorescein isothiocyanate; HPLC, high performance liquid chromatography; HRP, horseradish peroxidase; GGTase, geranylgeranyl transferase.

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Genetic construction of Rab24 and Rab1B in mammalian cells—The cDNA encoding Rab24 was obtained by PCR amplification using Taq DNA polymerase (Perkin-Elmer) and first strand cDNA template reverse-transcribed from mouse brain mRNA. Oligonucleotide primers for the PCR reaction were based on the published sequence of murine Rab24 (18). KpnI and BamHI restriction sites were added to the 5' and 3' primers respectively, to facilitate cloning of the PCR product in frame with a 5' sequence cassette encoding a Myc epitope (EQKLISEEDL), which we have introduced into the pCMV5 expression vector (19). The sequence of Rab24 was modified to encode a S→Q amino acid substitution at position 67, using overlap-extension PCR with appropriate mutator oligonucleotides and Pfu DNA polymerase (Stratagene Inc., La Jolla, CA). Constructs encoding Rab24 without the two histidine residues added to the COOH terminus (pCMVRab1B-1118) were generated from the corresponding wild-type DNA templates by PCR using appropriately modified 3' oligonucleotide primers. The sequences of all constructs were verified by DNA sequencing.

Assessment of the guanine nucleotide state of Rab proteins expressed in cultured cells—H-293 cells obtained from the American Type Culture Collection were grown in 60-mm dishes and transfected with 2 μg of the indicated pCMVRab construct, using LipofectAMINE Plus reagent (Life Technologies, Inc.) according to the instructions supplied by the manufacturer. Cultures were maintained in DMEM + 10% FCS for 24 h and then incubated for 5 h in fresh phosphate-free medium containing 100 μM [32P]orthophosphate (9000 Ci/mmol; NEN Life Science Products). Immunoprecipitation of radiolabeled Rab proteins, elution of bound [32P]-labeled guanine nucleotides, separation of GDP and GTP by thin layer chromatography, and determination of GDP/GTP ratios were carried out exactly as described previously (20).

Detection of endogenous Rab24 in various cell lines—An affinity-purified rabbit polyclonal antibody against Rab24 was generated by Zymed Laboratories Inc., using a synthetic peptide corresponding to the unique COOH-terminal hypervariable domain of Rab24 (amino acids 179–199), which was custom-synthesized by AnaSpec Inc., San Jose, CA.

To check the specificity of the Rab24 antibody, cDNA constructs encoding various Myc-tagged GTases were inserted into pET17b (Novagen) and recombinant proteins were expressed in Escherichia coli as described previously (21). Bacterial cell lysates were prepared in Buffer A (50 mM HEPES, pH 7.4, 5 mM MgCl2, 1 mM dithiothreitol, 100 μM GDP) and stored at −80 °C after addition of 20% v/v glycerol. Comparable amounts of the recombinant proteins were subjected to SDS-PAGE and immunoblot analysis using either the 9E10 mouse monoclonal anti-Myc antibody (Oncogene Sciences) or the Rab24 polyclonal antibody.

To assess the relative endogenous expression of Rab24 in mammalian cell lines, 293 cells, COS7, and N2a neuroblastoma cells were obtained from the American Type Culture Collection (Rockville, MD) and were grown in monolayer culture in a 5% CO2 atmosphere at 37 °C with DMEM + 10% FCS. Normal human fibroblasts were derived from a skin biopsy and maintained as described (22). Ntera2 teratocarcinoma cells were maintained in DMEM + 10% FBS and were induced to differentiate into mature post-mitotic neurons by treating the media with 20% (v/v) FCS and the cells were transfected with various pCMVRab constructs (4 μg of DNA) as described above. Beginning 1 h after transfection, prenylated proteins were metabolically labeled by adding fresh medium containing 200 μCi/ml [3H]mevalonolactone (3.4 Ci/mmol, American Radiochemical Corp.) and terminated after 1 h at 37 °C by addition of SDS sample buffer. Where indicated, lysates containing recombinant Rab proteins were supplemented with GDP (1 μM final concentration) and EDTA (7 μM final concentration) and preincubated at 25 °C for 10 min prior to being diluted 1:5 in the prenylation reaction. Incorporation of radioactivity into the Rab proteins was determined as described previously (27).

Geranylgeranylation of recombinant Rab24 in vitro—cDNA constructs encoding Myc-tagged Rab24 or Rab1B were subcloned into pET11a (Novagen, Madison, WI), and recombinant proteins were expressed in E. coli as described previously (21, 27). Bacterial cell lysates were prepared in Buffer A (50 mM HEPES, pH 7.4, 5 mM MgCl2, 1 mM dithiothreitol, 100 μM GDP) and stored at −80 °C after addition of 20% v/v glycerol. Expression of Myc-tagged recombinant proteins was verified by immunoblot analysis of E. coli lysates using the 9E10 mouse monoclonal anti-Myc antibody (Milab, San Diego, CA). HRP-labeled goat anti-mouse IgG was applied as the secondary antibody, and the bound IgG was detected by chemiluminescence using ECL reagent (Amersham Pharmacia Biotech). ECL signals were quantified by scanning the blots with a Lumi-Imager (Roche Molecular Biochemicals). Aliquots of E. coli lysate (10 μl) containing comparable amounts of each recombinant Rab protein (as determined by immunoblot assay) were added to reaction mixtures containing 50 mM HEPES, pH 7.4, 5 mM MgCl2, 1 mM dithiothreitol, 100 μM GDP, and 10 μl of a rat brain ammonium sulfate fraction enriched in geranylgeranytransferase activity, prepared as described (28). The final volume was 50 μl. All reactions were started by addition of 1 μl of [3H]GGPP (15 Ci/mmol, American Radiocemics Corp.) and terminated after 1 h at 37 °C by addition of SDS sample buffer. Where indicated, lysates containing recombinant Rab proteins were supplemented with GDP (1 μM final concentration) and EDTA (7 μM final concentration) and preincubated at 25 °C for 10 min prior to being diluted 1:5 in the prenylation reaction. Incorporation of radioactivity into the Rab proteins was determined as described previously (27).

Geranylgeranylation of Rab proteins in intact cells—To compare the properties of overexpressed Rab24 and Rab1B in H-293 cells, parallel 10-cm cultures were established in DMEM + 10% (v/v) FCS and the cells were transfected with various pCMVRab constructs (4 μg of DNA) as described above. Beginning 1 h after transfection, prenylated proteins were metabolically labeled by adding fresh medium containing 200 μCi/ml [3H]mevalonolactone (3.4 Ci/mmol) and 10 μM lovastatin. Cells were harvested after 18 h, and the expressed Myc-tagged Rab proteins were immunoprecipitated with the H12 epitope antibody (Invitrogen, Carlsbad, CA) and Super Signal chemiluminescent detection reagent. The relative incorporation of [3H]mevalonolactone (MVA) into each of the Myc-tagged proteins was determined by subjecting the remaining 90% of the immunoprecipitated protein to SDS-PAGE and fluorography as described previously (27).

Metabolic labeling of endogenous Rab proteins was performed by incubating IMR32 human neuroblastoma cells with [3H]mevalonolactone for 18 h, as described above. Parallel cultures were incubated under identical conditions, except that the medium was supplemented with [35S]methionine (100 μCi/ml, Trans-label, 1100–1200 Ci/mmol, ICN Pharmaceuticals Inc., Costa Mesa, CA). In one experiment, Rab1B and Rab24 were immunoprecipitated from equal aliquots of the same cell lysate. In a separate experiment, Rab6 and Rab24 were immunoprecipitated from the same cell lysate. The specific procedures used for immunoprecipitation, SDS-PAGE, and fluorography have been described (29). Films were scanned with a Molecular Dynamics densitometer, and the H and 35S signals were quantified as arbitrary units using ImageQuant software. To correct for variations in expression of different Rab proteins, the tritium values were normalized to the units of [35S]methionine-labeled Rab protein recovered from the parallel culture using the same antibody. The 35S values were adjusted to compensate for variations in the methionine content of each Rab protein.
supplemented with 0.15M NaCl, and 0.5-ml fractions were collected.

affinity-purified rabbit antibody against the Myc epitope (Upstate Biotechnologies), half of the membrane was immunoblotted with an affinity-purified polyacrylamide gels and transferred to PVDF membrane. The upper centrifugation, and disrupted in an equal volume of 100 mM Tris-HCl, three 100-mm cultures of IMR32 cells were harvested, pelleted by variation seen in Rab24 is the presence of a threonine instead plays a key role in GTP hydrolysis (33, 34). Another interesting variation seen in Rab24 is the presence of a threonine instead of aspartagine (Thr-120) in the NKD motif, which is one of four highly conserved sequence elements that contribute to the guanine nucleotide-binding pocket in Rab24 and most other Ras-related GTPases (35). The only other Ras-related proteins known to contain a threonine at this position are the members of the Cdc42/Rac family (36), which are functionally distinct from the Rab proteins (37). Two other features are worthy of note. First, Rab24 contains an insert with five consecutive arginine residues in the region corresponding to loop 8 of Ha-Ras, noted. First, Rab24 contains an insert with five consecutive arginine residues in the region corresponding to loop 8 of Ha-Ras, which are modified by geranylgeranyl moiety. The predicted amino acid sequence of Rab24 is compared with other Rab proteins and Ha-Ras. Amino acids marked with asterisks are unique in Rab24 and compared with all other Rab family members. Target cysteines for prenylation are undetected. Sequences and SWISS-PROT accession numbers are as follows: Rab24 (P52590), Rab1B (P10536), Rab3A (P20336), Rab5A (P18066), Rab6 (P20340), and Ha-Ras (P01112). Alignments were performed with ANPERTHON version 4.0 software by G. Deléage.

Interaction of Expressed Rab Proteins with FLAG-GDI in Transfected Cells—The ability of expressed Rab proteins to form stable complexes with epitope-tagged bovine brain Rab-GDI (GDIa) in transfected 293 cells was assessed by means of a co-immunoprecipitation assay described previously (21). Briefly, 293 cells growing in 10-cm dishes were co-transfected with plasmids encoding FLAG-GDIA and either Myc-Rab24 or Myc-Rab1B. After 24 h the cultures were harvested and cytosolic fractions were prepared. Ten percent of the cytosol was used to check for expression of FLAG-GDI and Myc-Rab proteins by immunoblot analysis. The remaining cytosol was incubated with 100 μl of a 20% suspension of anti-FLAG M2 affinity gel (IBI/Eastman Kodak Co.) and the bound FLAG-GDI protein complexes were eluted with 100 μl of 0.1 M glycine. The eluted proteins were resolved by SDS-PAGE in 11% polyacrylamide gels and transferred to PVDF membrane. The upper half of the membrane was immunoblotted with an affinity-purified rabbit antibody against the FLAG epitope (Zymed Laboratories Inc.), and the lower half of the membrane was immunoblotted with the affinity-purified rabbit antibody against the Myc epitope (Upstate Biotechnology, Inc., Saranac Lake, NY).

Gel Filtration Analysis of Endogenous Rab and GDI—Cells from three 100-mm cultures of IMR32 cells were harvested, pelleted by centrifugation, and disrupted in an equal volume of 100 mM Tris-HCl, pH 7.4, 1 mM MgCl₂, 0.1 mM GDP with complete mini-EDTA free protease inhibitor (Roche Molecular Biochemicals). A cytosolic fraction was prepared by centrifuging the cell lysate at 100,000 x g for 30 min. Gel filtration analysis was performed on 100 μl of cytosol, using a Beckman System Gold HPLC system equipped with a G2000SWXL 38-mm column (TosoHaas Inc., Montgomeryville, PA). Proteins were eluted at a flow rate of 1.0 ml/min, using the cell lysis buffer supplemented with 0.15 M NaCl, and 0.5-mM fractions were collected. Each fraction was mixed with 0.1 ml of 5× SDS sample buffer and one quarter of each fraction was subjected to SDS-PAGE and immunoblot analysis. The top portion of the PVDF membrane, containing proteins between 40 and 100 kDa, was incubated with an affinity-purified rabbit polyclonal antibody generated against amino acids 1–17 of bovine brain Rab GDI. This antibody recognizes the α (53 kDa) and β (46 kDa) forms of GDI, since the NH₂-terminal sequence is the same in both proteins (33). The lower portion of the PVDF membrane was immunoblotted with either the Rab1B polyclonal antibody (Zymed Laboratories Inc.) or the Rab24 antibody, as described earlier.

Immunofluorescent Localization of Myc-Rab24 and Endogenous Rab24—To visualize Myc-Rab24 by immunofluorescence, 293 cells were plated on laminin-coated coverslips in 60-mm dishes and transfected with pcMV-Rab24, using the calcium-phosphate method (31). After 18 h, the cells were fixed for 30 min in 4% (v/v) paraformaldehyde in PBS and permeabilized with 0.05% (v/v) Triton X-100 in PBS for 3 min. Immunofluorescence staining of the expressed Myc-Rab1B with the 9E10 anti-Myc antibody followed by FITC-conjugated goat anti-mouse IgG was performed as described previously (32). For staining of endogenous Rab24 or Rab1B in 293 cells or IMR32 neuroblastoma cells, essentially the same procedure was followed except that the affinity-purified anti-Rab24 rabbit IgG (10 μg/ml) or anti-Rab1B (Zymed Laboratories Inc.), was used as the primary antibody, with FITC-conjugated goat anti-rabbit IgG (Sigma) as the secondary antibody. In some experiments, the primary antibody solution was either omitted or pre-incubated for 1 h with the Rab24 peptide antigen at 15 μg/ml. Photomicrographs were taken with a Nikon Eclipse 800 fluorescence microscope equipped with a digital camera and imaging system (Phase 3 Imaging Systems, Glen Mills, PA).

RESULTS AND DISCUSSION

Rab24 Is Predominantly in the GTP-bound State When Expressed in 293 Cells—The predicted amino acid sequence of Rab24 contains several unique features not found in other members of the Rab family. This is illustrated by the sequence alignment in Fig. 1, where Rab24 is compared with Ha-Ras (for which the three-dimensional structure is known) and several other representatives of the Rab family. Of particular interest is the presence in Rab24 of a highly unusual serine in place of glutamine at position 67. The corresponding Gln-61 in Ha-Ras plays a key role in GTP hydrolysis (33, 34). Another interesting feature of Rab24 is the presence of a threonine instead of aspartagine (Thr-120) in the NKD motif, which is one of four highly conserved sequence elements that contribute to the guanine nucleotide-binding pocket in Rab24 and most other Ras-related GTPases (35). The only other Ras-related proteins known to contain a threonine at this position are the members of the Cdc42/Rac family (36), which are functionally distinct from the Rab proteins (37). Two other features are worthy of note. First, Rab24 contains an insert with five consecutive arginine residues in the region corresponding to loop 8 of Ha-Ras. In proteins of the Rho, Rac, and Cdc42 subfamily, this is the site of a larger structural element (~20 amino acids) termed the “Rho insert region” (38). Second, in Rab24, the two COOH-terminal cysteines, which are modified by geranylgeranyl moieties in other Rab proteins (39), are followed by two adjacent histidine residues, creating an unusual -CCHH motif that has not been observed in other prenylated proteins.

Introduction of a Q61L substitution into Rab reduces its intrinsic GTPase activity and renders the protein insensitive to GTPase-activating protein stimulation (40). Such Ras Gln-61 mutants are oncogenic, because they exist predominantly in the active GTP-bound state (33, 41). Analogous substitutions that have been created in Rab proteins by site-directed mutagenesis are known to cause similar reductions in GTPase activity (27, 42, 43). Most recently, Foster et al. (44) reported

FIG. 1. Sequence alignment of Rab24 with other Rab proteins and Ha-Ras. Amino acids marked with asterisks are unique in Rab24 and compared with all other Rab family members. Target cysteines for prenylation are undetected. Sequences and SWISS-PROT accession numbers are as follows: Rab24 (P52590), Rab1B (P10536), Rab3A (P20336), Rab5A (P18066), Rab6 (P20340), and Ha-Ras (P01112). Alignments were performed with ANPERTHON version 4.0 software by G. Deléage.
Atypical Properties of Rab24

that Rhoe, which is the only Ras-related protein besides Rab24 to possess a naturally occurring serine at the position cognate to Ras Gln-61, is unable to hydrolyze GTP or respond to Ras GTPase-activating protein. In light of these observations, we set out to determine how the presence of serine at position 67 in Rab24 might affect its guanine nucleotide state in intact cells. For comparison, we also determined the nucleotide state of Rab1B, which is typical of the Rab family insofar as it is found predominantly in the GDP state when overexpressed in mammalian cells (20). Different Myc-tagged Rab proteins were transiently overexpressed in HEK-293 cells, and the nucleotide pools were then labeled by incubating the cultures with [32P]orthophosphate. The thin layer chromatograms of the radiolabeled guanine nucleotides eluted from the immunoprecipitated Rab proteins show that Rab1B contained mainly GDP (Fig. 2). In contrast, the predominant nucleotide associated with Rab24 was GTP, consistent with very low GTPase activity.

To determine if the deficient GTPase activity of Rab24 could be attributed specifically to the presence of serine at position 67, the sequence of Rab24 was altered by site-directed mutagenesis so that Ser-67 was replaced with Gln, which occupies this position in all other known members of the Rab family. As shown in Fig. 2, this single amino acid substitution produced a substantial increase in Rab24 GTPase activity, as indicated by this position in all other known members of the Rab family. As 67, the sequence of Rab24 was altered by site-directed mutagenesis. As a result of this issue.

Deficient Preylation of Rab24—The geranylgeranylated COOH-terminal cysteine residues in the majority of Rab proteins are arranged in one of two common patterns; XXCC or XCXC. In a few cases (e.g., Rab5, Rab17), a CCXX motif occurs, but the presence of two histidine residues in the terminal X positions is unique to Rab24. This prompted us to evaluate the ability of Rab24 to undergo geranylgeranylation, using several different approaches. We began by determining the subcellular distribution of Myc-tagged Rab24 transiently expressed in HEK-293 cells. As shown in Fig. 3A, when the transfected cells were disrupted in buffer without detergent, Myc-Rab24 was found predominantly in the cytosol, with only 20% of the expressed protein recovered in the particulate fraction. Immunofluorescent staining of Myc-Rab24 showed a diffuse pattern throughout the cytoplasm, with some concentration in the perinuclear region (Fig. 3B). A similar pattern was described previously for Myc-Rab24 overexpressed in BHK and HeLa cells (18). To determine whether or not the small amount of Myc-Rab24 associated with the particulate fraction (Fig. 3A) might be prenylated, the cells were treated with lovastatin to block isoprenoid synthesis during the period immediately following transfection. This treatment typically depletes the pool of GGPP required for prenylation of Rab proteins and results in marked accumulation of unmodified Rab proteins in the cytosol, with a corresponding decrease in the membrane-associated Rab proteins (28). As shown in Fig. 3A, the relative amount of Myc-Rab24 associated with the particulate fraction in cells treated with lovastatin did not change compared with cells that were incubated without the drug. This observation provided the first hint that post-translational geranylgeranylation of Rab24 might be relatively inefficient compared with other Rab proteins and prompted a more detailed examination of this issue.

In the studies depicted in Fig. 4, similar amounts of recombinant Myc-tagged Rab24 or Rab1B were added to a cell-free system containing [3H]GGPP and a rat brain ammonium sulfate fraction enriched in protein:prenyltransferase activities. At the end of a 1-h reaction, incorporation of [3H]geranylgeranyl moieties into Rab24 was less than 10% of that observed with Rab1B (Fig. 4B). Previous studies have established that the enzyme complex responsible for prenylation of Rab proteins (RabGTPase) actually consists of a catalytic heterodimer termed geranylgeranyl transferase type II (GGTase II), and a carrier protein called Rab escort protein (REP) (45, 46). The Rab substrate must associate with REP before it can be geranylgeranylated by GGTase II (45, 47), and REP is known to interact preferentially with the GDP-bound form of the Rab protein (48). In earlier work with a GTPase-deficient Rab1B mutant, Rab1B(Q67L), we observed that the recombinant protein extracted from E. coli was a poor substrate for REP-GGTase II, probably because it remained in the GTP state. However, by preincubating Rab1B(Q67L) with a high concentration of GDP under conditions favorable for nucleotide exchange, we were able to restore its ability to be modified by GERanylgeranylation.

**FIG. 2. Guanine nucleotide content of Myc-Rab proteins expressed in HEK-293 cells.** Cells were incubated for 5 h with [32P]orthophosphate starting 24 h after transfection with the indicated constructs. Myc-tagged proteins were immunoprecipitated, and the 32P-labeled guanine nucleotides eluted from the proteins (15% of total volume) were subjected to thin layer chromatography along with GDP and GTP standards (upper panel). Radioactivity was quantified by scanning with a PhosphorImager. GDP/GTP ratios (with correction for difference in phosphate content of GDP versus GTP) were as follows: Myc-Rab24, 0.5; Myc-Rab1B, 29.8; Myc-Rab24(S67Q), 2.2; Myc-Rab1B(HH), 17.3; Myc-Rab24(HH), 0.3. Half of the remaining sample was subjected to SDS-PAGE and immunoblot analysis to compare the amounts of Myc-tagged protein recovered in each immunoprecipitate (lower panel).

**FIG. 3. Subcellular distribution of Myc-Rab24 expressed in HEK-293 cells.** A, parallel cultures of 293 cells were transfected with pCMVRab24 and incubated for 24 h with or without 10 μM lovastatin. Particulate (P) and soluble (S) fractions were prepared, and the proteins in each fraction were subjected to SDS-PAGE and immunoblot analysis using an antibody against the Myc epitope. Based on quantification of chemiluminescence signals with the Lumi-Imager, the particulate fraction contained 21.6% of the total Myc-Rab24 in the absence of lovastatin and 21.4% of the total in the presence of lovastatin. B, immunofluorescent staining of Myc-Rab24 in 293 cells. Cells were examined 24 h after transfection, using anti-Myc monoclonal antibody followed by FITC-conjugated goat anti-mouse IgG. The bar represents 10 μm.
Rab-GGTase in vitro (27). In light of these observations, we repeated the prenylation assays with recombinant Rab24 and Rab1B after a similar preincubation with GDP. In contrast to our earlier findings with Rab1B(Q67L), this treatment failed to improve the ability of Rab24 to undergo geranylgeranylation in the cell-free assay (Fig. 4C).

There is some evidence that the results of cell-free prenylation assays may not always accurately reflect the ability of Rab proteins to undergo prenylation in intact mammalian cells (21, 27). Therefore, the prenylation of Rab24 was also assessed by measuring the incorporation of the isoprenoid precursor, \([^{3}H]MVA\), into Myc-tagged proteins transiently expressed in 293 cells. The results of these metabolic labeling studies indicated that prenylation of Rab24 was markedly reduced compared with Rab1B (Fig. 5A). The difference in \([^{3}H]MVA\) incorporation was particularly striking when related to the amount of recovered Myc-tagged protein, which was much greater for Rab24 than for Rab1B. To further examine the potential connection between the deficient GTase activity of Rab24 and the poor prenylation of the expressed protein, a similar metabolic labeling study was conducted with Rab24(S67Q) (Fig. 5B). Prenylation of this mutant was not improved, despite the fact that its ability to hydrolyze GTP was much greater than that of the wild-type Rab24 (Fig. 2). This observation was consistent with the results of the cell-free assays with recombinant Rab24 (Fig. 4C), where preincubation of the protein with GDP failed to restore prenylation. Taken together, these findings raised the possibility that deficient prenylation of Rab24 was not due solely to its low GTase activity.

In considering other possible factors that might account for the inefficient prenylation of Rab 24, we turned our attention to the unusual COOH-terminal CCHH motif. To evaluate the potential role of the terminal histidine residues, we extended the normal COOH terminus of Rab1B (CC) by adding two histidines, thereby creating a CCHH motif identical to that found in Rab24, and, conversely, removed the two histidines from Rab24, to create a CC motif identical to that found in Rab1B. Neither of these COOH-terminal modifications altered the nucleotide state of the expressed proteins compared with their wild-type counterparts (Fig. 2). When Rab1B(±HH) was expressed in 293 cells, incorporation of \([^{3}H]MVA\) into the immunoprecipitated protein was drastically reduced compared with Rab1B(wt) (Fig. 5B). This implies that the presence of two histidine residues distal to the target cysteines may create a COOH-terminal conformation that is unfavorable for interaction with REP or GGTase II. However, this does not entirely explain the poor prenylation of Rab24 because, when we removed the COOH-terminal histidines from Rab24, prenylation was not restored to a level seen with Rab1B (Fig. 5B). Suspecting that the low intrinsic GTase of Rab24(−HH) could negate any beneficial effect of removing the histidines, we also removed the terminal histidines from the Rab24(S67Q) construct, which has a much higher GTase activity than Rab24wt (Fig. 2). However, prenylation of the Rab24(S67Q) in transfected cells was not improved by converting the COOH terminus to the classic XCCX motif (Fig. 5C). Thus, while the studies with Rab1B(+HH) indicate that the addition of two histidine residues distal to the target cysteines can hinder Rab prenylation, there must be other structural features in Rab24 that prevent the protein from serving as an efficient substrate for the Rab-GGTase, even when the COOH terminus conforms to a standard Rab XCCX motif.

Rab24 Does Not Associate with GDI—As mentioned in the Introduction, proteins termed Rab GDIs appear to play a key role in the cycling of GDP-bound Rab proteins between intracellular membranes and the cytosol. Rab proteins must be both geranylgeranylated and in the GDP conformation for optimal interaction with GDI (49–51). Since Rab24 has a low GTase activity and is not efficiently prenylated, we hypothesized that little if any of the expressed Rab24 generated in transfected 293 cells would be capable of associating with GDI. To test this hypothesis, we utilized an established assay in which Myc-tagged Rab proteins are transiently co-expressed with FLAG-tagged GDIa in transfected 293 cells and the FLAG-GDIa is subsequently collected from the cytosol on anti-FLAG affinity beads. As shown in Fig. 6, the expressed Myc-Rab24 and Myc-Rab1B proteins were readily detected in cytosol preparations from the 293 cells (Fig. 6A). However, only Myc-Rab1B was detected in the protein complexes eluted from the anti-FLAG affinity beads (Fig. 6B). The faint band seen in the samples from cells expressing Myc-Rab24 was nonspecific, since its was also observed when anti-FLAG beads were incubated with cytosol that did not contain FLAG-GDIa (Fig. 6B). Similar results were obtained when the experiment was performed with cells expressing FLAG-tagged GDI-2 (21, 52), which is representative of the GDIβ isoforms found in mammalian cells (data not shown). These findings indicate that Rab24 differs significantly from other Rab family members insofar as little if any of the expressed Rab protein enters the cytosolic GDI-bound pool.

Properties of Endogenous Rab24 in Cultured Cells—Previous work with a number of overexpressed epitope-tagged Rab proteins indicates that they retain functional specificity and exhibit subcellular distributions resembling those of their endogenous counterparts (20, 42, 53, 54). Nevertheless, because of the unusual properties of overexpressed Myc-Rab24 in 293 cells, we wished to confirm the preceding observations by studying the endogenous protein. RNA transcripts encoding Rab24 have been detected in a variety of mouse tissues, with the highest levels in the brain (18), but there is no available information about the expression of Rab24 in cultured cells. To facilitate these studies, we obtained a polyclonal antibody directed against a peptide sequence from the COOH-terminal hypervariable region of Rab24. This antibody reacted specifically with recombinant Rab24 in immunoblot assays and showed no significant cross-reactivity with Ha-Ras and several other Rab proteins (Fig. 7A). In addition, immunofluorescence assays indicated that the Rab24 antibody was as effective as...
analysis was performed using HRP-conjugated anti-Myc antibody and SuperSignal detection reagent. In the lower panel, one-tenth of each immunoprecipitate was subjected to SDS-PAGE and immunoblot analysis was performed using HRP-conjugated anti-Myc antibody and SuperSignal detection reagent. In the lower panel, the remainder of the immunoprecipitate was subjected to SDS-PAGE and fluorography to visualize the prenylated proteins (14-day exposure).

FIG. 5. Prenylation of Myc-Rab proteins expressed in HEK-293 cells. Cultures were transfected with each of the indicated Myc-Rab constructs. Immediately after transfection, cells were incubated with [3H]MVA for 18 h. The Myc-tagged proteins were immunoprecipitated as described under “Materials and Methods.” In the upper panel, one-tenth of each immunoprecipitate was subjected to SDS-PAGE and immunoblot analysis was performed using HRP-conjugated anti-Myc antibody and SuperSignal detection reagent. In the lower panel, the remainder of the immunoprecipitate was subjected to SDS-PAGE and fluorography to visualize the prenylated proteins (14-day exposure).

FIG. 6. Myc-Rab24 does not form a detectable complex with FLAG-GDI-α in 293 cells. Myc-Rab24 or Myc-Rab1B was transiently co-expressed with or without FLAG-GDI-α in HEK-293 cells as indicated below each panel. The FLAG-GDI complexes were collected from cell lysates using anti-FLAG affinity beads as described under “Materials and Methods.” Immunoblot analysis was performed on aliquots of cytosol (1/10) before addition of the anti-FLAG beads (A) or on the bound proteins eluted from the anti-FLAG affinity beads (B). The upper segment of each blot (45–66 kDa) was incubated with the anti-FLAG monoclonal antibody, while the lower segment (21–30 kDa) was incubated with the rabbit anti-Myc antibody. ECL reagent was used for chemiluminescent detection of bound secondary IgG.

FIG. 7. Specificity of the Rab24 antibody. A, aliquots of lysate from E. coli expressing the recombinant Myc-tagged protein indicated above each lane were subjected to SDS-PAGE and immunoblot analysis as described under “Materials and Methods,” using either the anti-Myc monoclonal antibody (upper panel) or the anti-Rab24 polyclonal antibody (lower panel). B, HEK-293 cells were transfected with pCMV-Rab24 and cells were subjected to immunofluorescence staining using either the anti-Myc monoclonal antibody or the anti-Rab24 polyclonal antibody as indicated. At the short exposure setting used for the micrographs, only the transfected cells show up clearly in the picture.

FIG. 8. Detection of endogenous Rab24 in various mammalian cell lines. A, equal amounts of protein (100 μg) from each cell line were subjected to SDS-PAGE and immunoblot analysis using an affinity-purified polyclonal antibody against Rab24 as described under “Materials and Methods.” B, immunoblot detection of Rab24 in undifferentiated teratocarcinoma stem cells (Ntera2) versus differentiated NT2N neurons derived from the stem cells by treatment with retinoic acid and mitotic inhibitors (23). All blots were developed with SuperSignal reagent.

The Myc antibody for detecting transfected cells that were transiently overexpressing Myc-Rab24 (Fig. 7B). In the stained cells, the diffuse cytoplasmic pattern seen with the Rab24 antibody was similar to that obtained with the Myc antibody.

To determine which cell lines might be most suitable for studies of endogenous Rab24, we used the Rab24 antibody to perform a comparative immunoblot analysis shown in Fig. 8. Among the cell lines tested, the highest levels of Rab24 were found in post-mitotic neurons (NT2N) generated by extended retinoic acid treatment of a human teratocarcinoma cell line (IMR32). The relatively high expression of Rab24 in the NT2N cells appeared to be directly related to the induction of neuronal differentiation, since the amount of Rab24 measured in the undifferentiated Ntera2 stem cells was only about one fourth of that detected in the post-mitotic neurons (Fig. 8B). In most of the cell types examined, the Rab24 antibody detected endogenous proteins that migrated as a closely-spaced doublet. Since cross-reaction of the Rab24 antibody with other Rab family members has not been observed (Fig. 7A), the most likely explanation for this observation would be the existence of more than one Rab24 isoform, or a posttranslational modification of the endogenous protein.

To determine whether endogenous Rab24 normally exists in a cytosolic complex with GDI, a detergent-free 100,000 x g supernatant fraction was prepared from IMR32 cells and subjected to gel filtration chromatography. Under these conditions Rab proteins typically are co-eluted with GDI at approximately 70–80 kDa, as expected for a 1:1 complex between Rab (25 kDa) and GDI (45 kDa) (50, 51, 55, 56). This is precisely what we observed when the column fractions were immunoblotted with antibodies that detect Rab1B or the α and β forms of GDI (Fig. 9). In contrast, when the column fractions were blotted with the Rab24 antibody, most of the immunodetectable protein was found in fractions that eluted well ahead of the known forms of GDI (Fig. 9). Thus, as suggested by our earlier studies...
of overexpressed Myc-Rab24, endogenous Rab24 in IMR32 cells seems to be largely excluded from the cytosolic GDI pool. The exception was a small amount of immunodetectable protein found in fractions 25 and 26, which consisted mainly of the upper band of the Rab24 doublet. This might represent a small pool of prenylated Rab24 associated with GDIβ in these fractions. Nearly identical results were obtained when cytosol was prepared from NT2N neurons (data not shown), indicating that the sequestration of Rab24 in a cytosolic complex separate from the GDI pool is not a cell type-specific phenomenon.

It is unclear why Rab24 was eluted from the column at approximately 100–120 kDa, since non-prenylated Rab3 proteins that fail to associate with GDI typically behave as 25-kDa monomers when subjected to gel filtration (20). One possibility is that Rab24 forms a stable cytosolic complex with REP. However, this seems unlikely, based on previous studies indicating that REP/Rab complexes elute at approximately 150–170 kDa (20, 57), which would place them in the void volume (fraction 17) in our study. An intriguing alternative is that Rab24 interacts with novel cytosolic protein partners not previously described for other members of the Rab family.

Fig. 10A shows the subcellular distribution of endogenous Rab24 in IMR32 neuroblastoma cells. Endogenous Rab24 was located predominantly in the soluble fraction. However, in accord with our earlier observations with the overexpressed Myc-Rab24 (Fig. 3A), approximately 20–25% of the endogenous Rab24 was found in the particulate fraction. To determine whether a significant percentage of endogenous Rab24 might be prenylated, the IMR32 cells were subjected to a widely used Triton X-114 phase-partitioning assay. In this assay, geranylgeranylated or farnesylated proteins are recovered in the particulate phase whereas non-prenylated proteins are retained in the aqueous phase (54, 58–60). The results depicted in Fig. 10B show that, as expected, significant amounts of Rab1B and Rab6 partitioned in the detergent phase. In contrast, Rab24 was detected only in the aqueous phase. To further assess the prenylation of endogenous Rab proteins, neuroblastoma cells were incubated with [3H]MVA and the incorporation of radioactivity into proteins immunoprecipitated with specific Rab antibodies was measured (Fig. 10C). By this method, the relative amount of isoprenoid precursor incorporated into Rab24 (normalized to the amount of [35S]methionine-labeled Rab24 recovered from a parallel culture) was approximately 25% of that observed for Rab1B or Rab6. Taken together, these findings strongly suggest that the inefficient prenylation of Myc-Rab24 initially observed in transfected cells (Figs. 3–5) reflects the properties of the endogenous cellular protein. It should be noted that the results of the Triton X-114-partitioning assay, which showed no Rab24 in the detergent phase (Fig. 10B), were not entirely consistent with the metabolic labeling studies (Fig. 10C), where incorporation of [3H]MVA into Rab24 was low but detectable. Although we cannot presently explain this discrepancy, one possibility is that the prenylated pool of Rab24 may be tightly associated with one or more novel proteins that mask the hydrophobic moiety, thereby preventing the protein from partitioning in the detergent phase.

As shown earlier (Figs. 3B and 7B), immunofluorescent localization of Myc-Rab24 in 293 cells revealed a diffuse staining pattern with a concentration in the perinuclear region. This pattern is quite similar to that originally described for Myc-Rab24 overexpressed in HeLa and BHK cells (18). Based on the partial overlap of Myc-Rab24 staining with markers for the endoplasmic reticulum, cis-Golgi, and endosomal compartments, Olikkonen and co-workers suggested that Rab24 might participate in an autophagic transport route that directs misfolded proteins from the endoplasmic reticulum to late endosomes or lysosomes. Additional evidence to support such a role is presently lacking. Moreover, since an antibody to Rab24 has not been available, there have been no studies of the subcellular localization of the endogenous cellular protein. The micrographs presented in Fig. 11 show the results obtained when 293 cells (Fig. 11A) and IMR32 neuroblastoma cells (Fig. 11D) were stained with our polyclonal antibody against Rab24. Although we observed a pattern of diffuse punctate staining similar to that seen with Myc-tagged Rab24, the most striking feature was the concentration of endogenous Rab24 in an array of discrete punctate structures distributed throughout the cytoplasmic compartment in both cell lines. The punctate distribution of Rab24 was quite different from the endoplasmic reticulum/Golgi pattern typically observed with a polyclonal

**Fig. 9.** Gel filtration assay to detect endogenous Rab-GDI complexes in IMR32 cells. A cytosolic fraction was prepared from IMR32 cells, and a 100-μl aliquot of cytosol was subjected to gel filtration on a column of Sephadex G-200. (A) The elution positions of chymotrypsinogen (25 kDa), bovine serum albumin (67 kDa), and alcohol dehydrogenase (150 kDa) or blue dextran (~150 kDa) are indicated at the top of the graph. Early fractions above 150 kDa did not contain immunoreactive proteins.

**Fig. 10.** Subcellular distribution and prenylation of endogenous Rab24 in neuroblastoma cells. A. IMR32 neuroblastoma cells were disrupted in buffer containing 1% Triton X-114 and subjected to the phase-partitioning assay described under "Materials and Methods." Equal aliquots of the detergent phase and aqueous phase were run on SDS gels and immunoblotted with antibodies to Rab1B, Rab6, or Rab24, as indicated above each panel. C, parallel cultures of IMR32 cells were incubated for 18 h with either [3H]MVA or [35S]methionine. Cell lysates were prepared from each culture and divided into equal aliquots. The indicated Rab proteins were immunoprecipitated and subjected to SDS-PAGE and fluorography. The graphs show the results of three separate experiments. In two experiments, Rab1B and Rab24 were immunoprecipitated from the same batch of radiolabeled cells. In a separate experiment, Rab6 and Rab24 were immunoprecipitated from the same cells. In each case, the amount of [3H]MVA incorporated into the specific immunoprecipitated Rab protein (arbitrary units derived from densitometric scans of the fluorograms) was normalized to the amount of [35S]-labeled Rab protein recovered from the parallel culture under identical conditions (see "Materials and Methods").
antibody raised against a peptide from the hypervariable region of Rab1B (Fig. 11B). Omission of the primary antibody (data not shown), or brief preincubation of the primary antibody with the Rab24 peptide antigen (Fig. 11C), eliminated the staining of the punctate structures, supporting the specificity of the Rab24 antibody staining. Inhibition of isoprenoid synthesis with 10 μM lovastatin for 48 h had no discernible effect on the number or intensity of Rab24-staining structures (Fig. 11, E and F), suggesting that the association of Rab24 with these structures does not depend on prenylation. The fact that overexpressed Myc-Rab24 seems to accumulate in the cytoplasm, rather than being concentrated in vesicular structures (Figs. 3B and 7B), may indicate that the latter structures contain Rab24 docking complexes or receptors that are saturated under normal physiological conditions. Alternatively, the conformation of the overexpressed Myc-Rab24 protein may differ in some critical aspect that prevents its correct subcellular targeting. It is worth noting that the small spherical structures reacting with the Rab24 antibody bear a strong resemblance to autophagic vacuoles described by others in MDCK cells and WI-38 fibroblasts (61). However, based on such preliminary morphological observations, it is premature to rule out the alternative possibilities that Rab24 is concentrated in secretory vesicles or endosomes. Studies are currently under way to further establish the nature of the Rab24-containing structures.

In conclusion, we have shown that Rab24 possesses at least three unique characteristics that distinguish it from other members of the Rab family. 1) Rab24 appears to have a very low GTPase activity in intact cells, primarily due to the presence of a serine instead of glutamine at the position cognate to Ras Gln-61. 2) Posttranslational geranylgeranylation of Rab24, which ends in a CCHH motif, is relatively inefficient when compared with “typical” Rab proteins that end with CXC and CC carboxyl-terminal motifs. 3) The majority of the Rab24 in the cytoplasmic compartment of cultured cells does not appear to associate with known forms of Rab GDI. These unusual characteristics suggest that if Rab24 does indeed participate in vesicular transport pathways involved in exocytosis, endocytosis or autophagy, it may operate through a novel mechanism that does not depend on GTP hydrolysis or GDI-mediated recycling.

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