Large Scale Screening for Novel Rab Effectors Reveals Unexpected Broad Rab Binding Specificity*

Mitsunori Fukuda†, Eiko Kanno, Koutaro Ishibashi, and Takashi Itoh

Small GTPase Rab is generally thought to control intracellular membrane trafficking through interaction with specific effector molecules. Because of the large number of Rab isoforms in mammals, however, the effectors of most of the mammalian Rabs have never been identified, and the Rab binding specificity of the Rab effectors previously reported has never been thoroughly investigated. In this study we systematically screened for novel Rab effectors by a yeast two-hybrid assay with 28 different mouse or human Rabs (Rab1–30) as bait and identified 27 Rab-binding proteins, including 19 novel ones. We further investigated their Rab binding specificity by a yeast two-hybrid assay with a panel of 60 different GTP-locked mouse or human Rabs. Unexpectedly most (17 of 27) of the Rab-binding proteins we identified exhibited broad Rab binding specificity and bound multiple Rab isoforms. As an example, inositol-polyphosphate 5-phosphatase OCRL (oculocerebrorenal syndrome of Lowe) bound the greatest number of Rabs (i.e. 16 distinct Rabs). Others, however, specifically recognized only a single Rab isoform or only two closely related Rab isoforms. The interaction of eight of the novel Rab-binding proteins identified (e.g. INPP5E and Cog4) with a specific Rab isoform was confirmed by co-immunoprecipitation assay and/or colocalization analysis in mammalian cell cultures, and the novel Rab2B-binding domain of Golgi-associated Rab2B interactor (GARI) and GARI-like proteins was identified by deletion and homology search analyses. The findings suggest that most Rab effectors (or Rab-binding proteins) regulate intracellular membrane trafficking through interaction with several Rab isoforms rather than through a single Rab isoform. Molecular & Cellular Proteomics 7: 1031–1042, 2008.

The Rab family belongs to the Ras-like small GTPase superfamily, and it is thought to be involved in the control of intracellular membrane trafficking in all eukaryotic cells (for reviews, see Refs. 1–3). Like other G proteins, Rab cycles between two nucleotide-bound states, a GDP-bound state and a GTP-bound state, and the GTP-bound active form of Rab promotes membrane trafficking through interaction with specific effector molecules (1–3). Thus, identification of the specific Rab effector molecules is one of the most important steps toward understanding the molecular mechanisms of Rab-mediated membrane trafficking. However, because a large number of Rab isoforms have been reported in mammals (more than 60 in humans and mice) (4–7), identification of Rab effectors has for the most part been limited to the Rabs that are conserved from yeasts to mammals (e.g. Rab1/Ypt1, Rab5/Ypt5, Rab6/Ypt6, Rab7/Ypt7, and Rab11/Ypt31). In addition, with the exception of Rab27 effectors (8–10), the Rab binding specificity of the mammalian Rab effectors that have been described previously has never been thoroughly investigated, suggesting the possibility that they bind other Rabs that have never been investigated. Consistent with this notion, rabphilin, which was originally identified as a specific Rab3 effector (11), and Rabenosyn-5, which was originally identified as a Rab5 effector (12), have recently been reported to also bind other Rab isoforms (8, 9, 13), indicating the importance of reassessment of the Rab binding specificity of previously described Rab effectors.

In this study we exhaustively screened for novel Rab effectors by a yeast two-hybrid assay with Rab1–Rab30 as bait and thoroughly investigated their Rab binding specificity by using a panel of 60 different GTP-locked Rabs (7). Unexpectedly approximately two-thirds (17 of 27) of the Rab-binding proteins we identified exhibited broad Rab binding specificity and bound multiple Rab isoforms, whereas the others recognized only a single Rab isoform (or members of a single Rab subfamily). Based on these findings, we discuss the specificity and diversity of Rab-effector interactions in membrane trafficking.

MATERIALS AND METHODS

Yeast Two-hybrid Screening—Sixty different GTP-locked mouse or human Rab cDNAs (Rab1–43; GenBank™/EBI accession numbers AB232583–AB23642) were prepared as described previously (7). To promote efficient targeting of the bait-prey complex into the yeast nucleus in the two-hybrid assay, C-terminal Cys residues that are known to undergo geranylation were removed or replaced with Ala by PCR using the following oligonucleotides (stop codons in bold): 5'-TCA GCT CTC CAC CTG ACT GCT-3' (Rab1AΔCys primer, antisense); 5'-TCA GCC ACC GCT AGC AGG CT-3' (Rab1BΔCys primer,
Large Scale Screening for Novel Rab Effectors

The cDNA of the C-terminal coiled-coil domain of mKIAA0819 (amino acid residues 940–1121) and of mKIAA0903 (amino acid residues 1014–1231) was amplified by PCR from Marathon-Ready adult mouse testis cDNA using the following oligonucleotides with a restriction enzyme site (underlined; BamHI or BglII) or a stop codon (boldface) as described previously (15): 5'-GGATCCGGAGAAGGACCTGGATGAGAGG-3' (mKIAA0819 Met primer, sense) and 5'-TCAAGGTTCCGTCCTCGTCTGAG-3' (mKIAA0819 stop primer, antisense). Purified PCR products were directly inserted into the pGEM-T Easy vector (Promega, Madison, WI). The cDNA inserts were sequenced by an automated sequencer and then subcloned into the pGAD-C1 vector (14). The Rab binding specificity of mKIAA0819 and mKIAA0903 was investigated by using the panel of 60 different Ras as described above.

Molecular Cloning of Full-length Rab-binding Proteins by PCR—Because most of the clones we identified by yeast two-hybrid screening contained a fragment of Rab-binding proteins (supplemental Fig. S1), the full-length cDNA of some of these proteins (i.e. A5076111) was amplified by PCR from Marathon-Ready adult mouse testis cDNA, 2% Bacto agar, 0.01% uracil, 0.0125% lysine, and 0.01% sodium lacking adenine, histidine, leucine, and tryptophan (SC-AHL medium; 0.67% yeast nitrogen base without amino acids, 2% glucose, 2% Bacto agar, 0.01% uracil, 0.125% lysine, and 0.01% methionine) as a selection medium. All reagents used in this study were analytical grade or the highest grade commercially available.

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Large Scale Screening for Novel Rab Effectors

To identify novel Rab effectors, we systematically screened mouse testis and mouse embryo cDNA libraries (~1 x 10^9 colonies for each Rab) by using the following 28 different pGBD-Rabs as bait: Rab1B, Rab2B, Rab4B, Rab6C, Rab8B, Rab9A/B, Rab10, Rab11B, and Rab12–30. Although no positive clones were obtained for Rab4B, Rab6C, Rab18, Rab21, Rab22A, Rab23, Rab26, or Rab28, 27 different Rab-binding proteins were obtained for other Rabs (supplemental Fig. S1). In addition to previously well characterized Rab effector proteins (e.g. Rab11-FIP2 (19) and Syt1/Slp1 (8–10)) as well as Rab regulatory enzymes (e.g. Evi5 with a Tre-2/Bub2/ Cdc16 Rab-GAP domain (7, 20) and Rab3ip/Rabin3 with a Sec2 guanine nucleotide exchange factor domain (21, 22)) (supplemental Fig. S2), 19 previously uncharacterized Rab-binding proteins were obtained. The Rab binding specificity of these positive clones (indicated by asterisks in supplemental Fig. S1) was investigated by using the panel of 60 different GTP-locked Rabs (summarized in Table I). To our surprise, most (17 of 27) of the Rab-binding proteins exhibited broader Rab binding specificity than we had previously thought and bound multiple (up to 16) distinct Rab isoforms. By contrast, AI507611 (referred to as GARI below), Nde1, Wdr38, INPP5E, and Cog4 each specifically recognized only a single Rab isoform (see Table I for details). All of the Rab-binding proteins were further classified into several groups in terms of their putative functions and/or their domain structures (see below for details).

**Broad Rab Binding Specificity of Molecules Interacting with CasL (MICALs) and MICAL-like Proteins—** MICAL-1 and MICAL-like proteins (MICAL-Ls) form a family of evolutionarily conserved signal transduction proteins that shares a calponin homology (CH) domain, LIM domain, and C-terminal coiled-coil (CC) domain (23, 24) (Fig. 1A). The C-terminal CC domain

**Immunofluorescence Analysis—** pEGFP-C1-Rabs (6) and pmRFP-C1-Rab-binding proteins were coexpressed in PC12 cells by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Two days after transfection, cells were harvested and fixed with 4% paraformaldehyde (Wako Pure Chemicals, Osaka, Japan) for 20 min. Cells were then analyzed with a confocal laser-scanning microscope (Fluoview FV500; Olympus, Tokyo, Japan), and the images were processed with Adobe Photoshop software (version 7.0).
is highly conserved between MICAL-1 and MICAL-Ls (Fig. 1B) and functions as a protein interaction site (e.g. the MICAL-1 CC domain interacts with vimentin (23) and plexin A (24)). MICAL-C-terminal-like (cl) contains only the conserved CC domain and lacks both the CH domain and LIM domain. MICAL-1 and MICAL-L2/JRAB (junctional Rab13-binding protein) have recently been shown to bind Rab1 and Rab13, respectively, through their CC domain (25–27), but their Rab binding specificity has never been thoroughly investigated, suggesting the possibility that MICALs and MICAL-Ls also bind Rabs other than Rab1 and Rab13. Actually the results of an exhaustive analysis with the Rab panel (7) indicated that MICAL-1, MICAL-cl, MICAL-L1, and MICAL-L2 all bind at least five distinct Rabs (i.e. Rab8A/B, Rab10, Rab13, and Rab15, all of which are phylogenetically similar (5, 9)) and that the Rab binding specificity of the four proteins was slightly different (Table I and Fig. 1C). All of these Rabs are likely to bind to the same site (i.e. CC domain) in both MICALs (MICAL-1 and MICAL-cl) and MICAL-Ls because all of the clones we identified contained the C-terminal CC domain (supplementary Fig. S1). The most striking difference between MICALs and MICAL-Ls is that only MICALs possess Rab1A/B binding ability. The above findings suggest that MICALs and MICAL-Ls may play redundant or similar roles in the post-Golgi or endocytic trafficking pathway (27) when they bind Rab8A/B, Rab10, Rab13, or Rab15 and that MICALs might have an additional role in membrane trafficking in the Golgi when they bind Rab1A/B (25).

A homology search analysis revealed that two additional proteins, mKIAA0819 and mKIAA0903, contain a similar CC domain in their C-terminal domain (Fig. 1A, parentheses). Although neither of these proteins was identified by our yeast two-hybrid screening, it is highly possible that the CC domains of mKIAA0819 and mKIAA0903 function as certain Rab-binding domains. To investigate this possibility, we cloned the C-terminal CC domains of mKIAA0819 (amino acid residues 940–1121) and mKIAA0903 (amino acid residues 1041–1231) and investigated their Rab binding ability by a yeast two-hybrid assay. As anticipated, the mKIAA0819 CC domain interacted strongly with Rab8A, Rab10, Rab15, and Rab35 and weakly with Rab1A, Rab8B, and Rab13 (supplemental Fig. S3, left panel), the same as

| Names of Rab-binding proteins | Gene ID | Rab binding specificity determined in this study | Rab binding specificity reported previously |
|------------------------------|--------|-----------------------------------------------|--------------------------------------------|
| Cog6                         | 67542  | Rab1A/B, -6A/B, -41                            | Rab1A/B, -5A, -6A, -8A, -14 (28)           |
| OCRL                         | 320634 | Rab1A/B, -3A/B/D, -5A/B/C, -6A/B, -8A/B, -13, -22B, -35 |                                    |
| Gmc1                         | 23885  | Rab2B                                         |                                             |
| Al507611/GARI                | 245884 | Rab2B                                         |                                             |
| OTTMUSG00000005491/GARI-L3  | 432552 | Rab2A/B                                       |                                             |
| Nde1                         | 67203  | Rab9A/B                                       |                                             |
| Ywha/14-3-3¢                 | 22630  | Rab9A/B                                       |                                             |
| SmcD1                        | 74355  | Rab8A/B, -10, -11A/B, -12, -25, -29, -36, -41  |                                             |
| Evl5                         | 14020  | Rab8A/B, -10                                 |                                             |
| Rab3ip                       | 216363 | Rab11A/B                                      | Rab3a/D, -8 (21, 22)                       |
| 2900002H16Rik                | 75695  | Rab12, -34, -36, -40B                        |                                             |
| MICAL-1                      | 171580 | Rab1A/B, -8A/B, -10, -13, -15, -35, -36       | Rab1 (25)                                  |
| MICAL-cl                     | 70877  | Rab1A/B, -8A/B, -10, -13, -15, -35, -36       | Rab1 (26)                                  |
| MICAL-like2                  | 231830 | Rab8A/B, -10, -13, -15                       | Rab13 (27)                                 |
| MICAL-like1                  | 27008  | Rab8A/B, -10, -13, -15, -35, -36             |                                             |
| Akap10                       | 56697  | Rab4A/B, -14, -19, -40A/B/C, -41a            |                                             |
| 493057319Rik                 | 104859 | Rab5A/B/C, -17, -22B, -24, -29, -38a         |                                             |
| Osgin2                       | 209212 | Rab15, -19                                    |                                             |
| Wdr38                        | 76646  | Rab19                                         |                                             |
| INPP5E                       | 64436  | Rab20                                         |                                             |
| INPP5B                       | 16330  | Rab1A/B, -3A/B/D, -5A/B/C, -6A/B, -8A/B, -9A, -13, -22B  |
| CtbP1                        | 13016  | Rab24                                         | Rab1, -2, -5, -6, -9 (29)                   |
| Rab11-FIP5                   | 52055  | Rab11A/B, -25                                 |                                             |
| Rab11-FIP2                   | 74998  | Rab11A/B, -14, -25                            | Rab11A/B, -25 (19)                         |
| Syt1/Slp1                    | 269589 | ND                                            | Rab27B/A (8–10)                            |
| Golg4                        | 54214  | Rab27B/a, -30, -41                            |                                             |
| Cog4                         | 102339 | Rab30                                         |                                             |

a Weak interaction in the yeast two-hybrid assay.
b Not determined.
MICALs did (Fig. 1C). By contrast, however, the mKIAA0903 CC domain did not bind any Rab isoforms (supplemental Fig. S3, right panel).

**Broad and Specific Rab Binding Activity of Inositol-polyphosphate 5-Phosphatases—** Three inositol (or phosphatidylinositol)-polyphosphate 5-phosphatases, which are...
Fig. 2. Structure of inositol-polyphosphate 5-phosphatases that bind Rab isoforms. A, domain structure of mouse OCRL, INPP5B, and INPP5E. OCRL and INPP5B share an IPP domain (shaded boxes) and C-terminal Rho-GAP domain (hatched boxes), whereas INPP5E contains only the IPP domain. Amino acid numbers are indicated on both sides. B, sequence alignment of the putative Rab-binding domain of mouse (Mm) OCRL (28), mouse INPP5B (29), and their invertebrate homologues (Dm, Drosophila; and Ce, C. elegans). Residues that are conserved in more than three of the sequences and residues that are similar in more than three of the sequences are shown against a black background and against a shaded background, respectively. Amino acid numbers are indicated on the right. C, specific interactions between OCRL, INPP5B, and INPP5E and Rab5 as revealed by the yeast two-hybrid assay. Yeast cells containing pGBD plasmid expressing GTP-locked Rab lacking the C-terminal Cys residues (positions indicated in the left panel) and pAct2 plasmid expressing inositol polyphosphatases were streaked on SC-AHLW medium and incubated at 30°C. Note that INPP5E specifically bound Rab20, whereas OCRL and INPP5B each bound 16 distinct Rab isoforms. D, colocalization between INPP5E (red) and Rab20 (green) in the Golgi (GM130; blue) of PC12 cells. Scale bar,
Mg²⁺-dependent Li⁺-sensitive enzymes, were identified as Rab-binding proteins (Fig. 2). Oculocerebrorenal syndrome of Lowe (OCRL) and inositol-polysphosphate 5-phosphatase B (INPP5B; or 72-kDa inositol-polysphosphate 5-phosphatase) are highly homologous to each other and share both an inositol-polysphosphate phosphatase (IPP) domain (Fig. 2A, shaded boxes) and a C-terminal Rho-GAP domain (hatched boxes). By contrast, INPP5E contains only the IPP domain and lacks a Rho-GAP domain. Although OCRL has been shown recently to bind several Golgi-associated Rabs and endosomal Rabs (e.g. Rab6A and Rab5A) (28), OCRL unexpectedly exhibited the broadest Rab binding specificity among the Rab-binding proteins identified in this study, having bound 16 distinct Rabs (Fig. 2C, left panel). The Rab-binding site of OCRL has recently been mapped to the region between the IPP domain and Rho-GAP domain (amino acid residues 480–709) (28) (supplemental Fig. S1). The putative Rab-binding site has been conserved from invertebrates (Caenorhabditis elegans and Drosophila melanogaster) to vertebrates and is also retained in INPP5B (Fig. 2B). Consistent with this high sequence conservation, INPP5B also bound 16 distinct Rabs but with slightly different Rab binding specificity from that of OCRL (Fig. 2C, middle panel). While preparing this manuscript, the Rab binding ability of INPP5B was also shown to be important to its targeting of the Golgi apparatus or endosomes (29).

In contrast to OCRL and INPP5B, INPP5E specifically bound Golgi-localized Rab20 (30) in our yeast two-hybrid assay (Fig. 2C, right panel). Actually INPP5E and Rab20 were co-localized in the Golgi (i.e. GM130) of PC12 cells (Fig. 2D), and their interaction was further confirmed by co-immunoprecipitation assay (Fig. 2E). Unlike OCRL and INPP5B (28, 29), however, INPP5E preferentially bound the GDP-bound form of Rab20 over its GTP-bound form (Fig. 2E, middle panel, lanes 1 and 2). Similarly INPP5E prefered a CN mutant of Rab20 to its CA mutant (data not shown). Although INPP5E is present in the Golgi (31), the same as OCRL is, INPP5E does not contain the above mentioned Rab-binding region that was found in OCRL, indicating the presence of a novel Rab-binding site in INPP5E. By analogy to the Rab binding ability of OCRL in its targeting to the Golgi or endosomes, we speculate that the Pro-rich domain, which has been shown to be required for the Golgi localization of INPP5E (31), contains a novel Rab20-binding domain. As anticipated, the N-terminal Pro-rich domain (INPP5E-N; amino acid residues 1–300 of INPP5E) alone was sufficient for Rab20 binding activity (Fig. 2E, middle panel, lanes 5 and 6), the same as the full-length protein.

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10 μm. E, interaction between INPP5E and Rab20 in COS-7 cells. Agarose beads coupled with FLAG-Rab20 were incubated with COS-7 cell lysates containing T7-INPP5E (lanes 1 and 2) and T7-INPP5E-N (lanes 5 and 6) in the presence of 0.5 mM GTP-S and 1 mM GDP, respectively. After washing the beads, proteins bound to the beads were analyzed by 10% SDS-PAGE followed by immunoblotting with HRP-conjugated anti-T7 and anti-FLAG tag antibodies. Note that both INPP5E and INPP5E-N preferentially bound the GDP-bound form of Rab20 (middle panel, lanes 2 and 6). Molecular mass markers (1 x 10⁻²) are shown on the left. IP, immunoprecipitate; T, GTP-S; D, GDP.
Fig. 3. Structure of protein components of the Golgi complex that bind Rab isoforms. A, domain structures of mouse Cog4, Cog6, and Golga4. These three proteins do not resemble each other, but all of them contain one or more CC domains (black boxes). Shaded boxes and the hatched box indicate the Cog4/Cog6 domain and the Grip domain, respectively. Note that the CC domain of Cog6 is part of the Cog6 domain according to the results of a SMART analysis. Amino acid numbers are indicated on both sides. B, specific interaction between Cog4, Cog6, and Golga4 and Rabbs as revealed by the yeast two-hybrid assay. Yeast cells containing pGDB plasmid expressing GTP-locked Rab lacking the C-terminal Cys residues (positions indicated in the left panel) and pAct2 plasmid expressing protein components of the Golgi complex were streaked on SC-AHLW medium and incubated at 30 °C. Note that Cog4 specifically bound Rab30. C, colocalization between Cog4 or Cog4-N (red) and Rab30 (green) in the Golgi (GM130; blue) of PC12 cells. Scale bars, 10 μm. D, interaction between Cog4 and Rab30 in COS-7 cells. Agarose beads coupled with FLAG-Rab30 were incubated with COS-7 cell lysates containing T7-Cog4 (lanes 1 and 2) and T7-Cog4-N (lanes 5 and 6) in the presence of 0.5 mM GTPγS and 1 mM GDP, respectively. After washing the beads, proteins bound to the beads were analyzed by 10% SDS-PAGE followed by immunoblotting with HRP-conjugated anti-T7 and anti-FLAG tag antibodies. Note that Cog4 preferentially bound the GDP-bound form of Rab30 (middle panel, lanes 2 and 6). Molecular mass markers (×10^3) are shown on the left. IP, immunoprecipitate; T, GTPγS; D, GDP; *, non-specific bands of IgG used for immunoprecipitation.

bound form) and weakly with Rab34 in a co-immunoprecipitation assay (Fig. 4C), 2900002H16Rik is likely to function as a Rab12 effector. AKap10 was originally identified as an A kinase-anchoring protein containing two regulator of G protein signaling domains (33), which are thought to control G protein-coupled receptor signaling (supplemental Fig. S1). Because the AKap10 clone we identified contained the C-terminal half of the protein, the second regulator of G protein signaling domain may function as a binding domain for some endosomal Rabs (e.g., Rab4A/B, Rab14, Rab19, and Rab40A/B/C; Fig. 4A, right middle panel). 4930573I19Rik is a large as yet uncharacterized protein composed of 1423 amino acids, and it contains three WD repeats at the N terminus and 11 TECPR domains (β propeller repeats in Physarum polycephalum tectonins, Limulus lectin L-6, and animal hypothetical proteins) at the C terminus (supplemental Fig. S1). 4930573I19Rik strongly bound six Rabs, Rab5A/B/C, Rab17, Rab22B, and Rab24 (Fig. 4A, right panel), most of which are involved in endosomal trafficking. Because the 4930573I19Rik clone we identified lacked N-terminal WD repeats, these Rabs presumably bind C-terminal TECPR domains or some other as yet uncharacterized region.

Putative Rab Effector Proteins That Bind a Single Rab Isoform—Additional putative Rab effector proteins that specifically recognize a single Rab isoform or only two closely related Rab isoforms were identified by our yeast two-hybrid assay (Fig. 4B and Table I). OTTMUSG00000005491, AI507611, and germ cell-less homolog 1 (Gmcl1) interacted specifically with Rab2B; Nde1 and Ywhaq/14-3-3 interacted with Rab9A/B; Osgim2 interacted with Rab15 and Rab19; Wdr38 interacted with Rab19; and CtBP1 interacted with Rab24, and some of their
FIG. 4. Putative Rab effector proteins that bind multiple Rab isoforms (A) or a single Rab isoform (B) as revealed by the yeast two-hybrid assay are shown. Yeast cells containing pGBD plasmid expressing GTP-locked Rab lacking the C-terminal Cys residues (positions indicated in the left panel) and pAct2 plasmid expressing the indicated proteins were streaked on SC-AHLW medium and incubated at 30 °C. C, interaction between 2900002H16Rik and Rab12 or Rab34 in COS-7 cells. D, interaction between Rab9B and Nde1, Rab19 and Wdr38, and Rab24 and CtBP1. Agarose beads coupled with FLAG-Rab(CA) and -Rab(CN) mutant were incubated with COS-7 cell lysates containing T7-Nde1 (lanes 1 and 2), T7-Wdr38 (lanes 3 and 4), or T7-CtBP1 (lanes 5 and 6) in the presence of 0.5 mM GTP and 1 mM GDP, respectively. After washing the beads, proteins bound to the beads were analyzed by 10% SDS-PAGE followed by immunoblotting with HRP-conjugated anti-T7 and anti-FLAG tag antibodies. Note that CtBP1 preferentially bound the CA mutant of Rab24 (mimics GTP-bound form) (middle panel, lane 5), whereas Wdr38 preferentially bound the CN mutant of Rab19 (mimics GDP-bound from) (middle panel, lane 4). By contrast, Nde1 bound both the CA mutant and the CN mutant of Rab9B (middle panel, lanes 1 and 2), although the Nde1-Rab9B interaction appeared to be weak. Molecular mass markers are shown on the left. IP, immunoprecipitate; *, non-specific bands of IgG used for immunoprecipitation.
interactions were confirmed by co-immunoprecipitation assay (Fig. 4D). We especially focused on one of these putative Rab effector proteins, AI507611, a novel Rab2B-binding protein in the Golgi (Fig. 5C) that we have referred to here as GARI-L1-L5. The homology plot analysis of the N-terminal domains of GARI and OTTMUSG00000005491 (referred to as GARI-L3 below), another Rab2B-binding protein identified in this study (Fig. 5A, shaded boxes), and both proteins bound a constitutive active mutant of Rab2B (mimics the GTP-bound form) in the co-immunoprecipitation assay (Fig. 5D, lanes 1–4), suggesting that they share a common Rab2B effector domain. Second, a standard protein BLAST search revealed four additional previously uncharacterized GARI-related proteins (GARI-L1/2/4/5). We therefore attempted to identify their Rab2B-binding domain by deletion analysis and sequence alignment of the putative Rab2B-binding domain of GARI and GARI-L1-L5. Residues that are conserved in more than half of the sequences and residues that are similar in more than half of the sequences are shown against a black background and against a shaded background, respectively. Amino acid numbers are indicated on both sides. B, sequence alignment of the putative Rab2B-binding domain of GARI and GARI-L1–L5. Residues that are conserved in more than half of the sequences are indicated on both sides. Scale bar, 10 μm. C, colocalization between GARI (red) and Rab2B (green) in the Golgi (GM130; blue) of PC12 cells. Scale bar, 1 μm. D, interaction between Rab2B and GARI (open arrowhead) and GARI-L3 (closed arrowhead) in COS-7 cells. Note that the N-terminal domain of GARI-L3 (GARI-L3-N, open arrow), but not the C-terminal domain (GARI-L3-C, closed arrow), is sufficient for interaction with Rab2B in COS-7 cells (lanes 5–8). Agarose beads coupled with T7-GARI (lanes 1 and 2) or T7-GARI-L3 (lanes 3–8) were incubated with COS-7 cell lysates containing the FLAG-Rab(CA) mutant in the presence of 0.5 mM GTPγS and FLAG-Rab(CN) mutant in the presence of 1 mM GDP. After washing the beads, proteins bound to the beads were analyzed by 10% SDS-PAGE followed by immunoblotting with HRP-conjugated anti-T7 and anti-FLAG tag antibodies. Molecular mass markers (×10^5) are shown on the left. IP: immunoprecipitate; N, GARI-L3-N; C, GARI-L3-C.

Fig. 5. Characterization of the novel Rab2B-binding domain. A, domain structures of mouse GARI and related proteins GARI-L1–L5. The putative Rab2B-binding site is shown as shaded boxes (RBD2B). GARI/AI507611 and GARI-L3/OTTMUSG00000005491 were identified as Rab2B-binding proteins in this study. GARI-L1/NYD-SP18, GARI-L2/4921509E07Rik, GARI-L4/4933417M04Rik, and GARI-L5/1700021P22Rik were obtained by a standard protein BLAST search using GARI and GARI-L3 as bait. Amino acid numbers are indicated on both sides. B, sequence alignment of the putative Rab2B-binding domain of GARI and GARI-L1-L5. Residues that are conserved in more than half of the sequences and residues that are similar in more than half of the sequences are shown against a black background and against a shaded background, respectively. Amino acid numbers are indicated on the right. C, colocalization between GARI (red) and Rab2B (green) in the Golgi (GM130; blue) of PC12 cells. Scale bar, 10 μm. D, interaction between Rab2B and GARI (open arrowhead) and GARI-L3 (closed arrowhead) in COS-7 cells. Note that the N-terminal domain of GARI-L3 (GARI-L3-N, open arrow), but not the C-terminal domain (GARI-L3-C, closed arrow), is sufficient for interaction with Rab2B in COS-7 cells (lanes 5–8). Agarose beads coupled with T7-GARI (lanes 1 and 2) or T7-GARI-L3 (lanes 3–8) were incubated with COS-7 cell lysates containing the FLAG-Rab(CA) mutant in the presence of 0.5 mM GTPγS and FLAG-Rab(CN) mutant in the presence of 1 mM GDP. After washing the beads, proteins bound to the beads were analyzed by 10% SDS-PAGE followed by immunoblotting with HRP-conjugated anti-T7 and anti-FLAG tag antibodies. Molecular mass markers (×10^5) are shown on the left. IP: immunoprecipitate; N, GARI-L3-N; C, GARI-L3-C.
comparison between GARI and GARI-Ls (Fig. 5B). As shown in Fig. 5D (lanes 5–8), the N-terminal domain of GARI-L3 alone was necessary and sufficient for binding to Rab2B, and the large C-terminal domain of GARI-L3 was not. Thus, the N-terminal domain of GARI and GARI-Ls is likely to function as a Rab2B effector domain that modulates the function of Rab2B in the membrane trafficking in the Golgi.

DISCUSSION

Small GTPase Rab is generally thought to be an essential regulator of intracellular membrane trafficking through interaction with its specific effector molecule(s). Because of the large number of Rab isoforms in mammals (4–7), however, specific effector molecules for most of the mammalian Rabs have never been elucidated, and even the Rab binding specificity of the previously characterized Rab effectors has never been thoroughly investigated. In the present study we for the first time performed large scale screening for novel Rab effectors by a yeast two-hybrid assay with Rab1–Rab30 as bait and determined their Rab binding specificity by using the panel of 60 different GTP-locked Rabs (7). As a result, we were able to identify 27 Rab-binding proteins, including 19 novel ones (Table I). One of the most surprising findings in this study is that 17 of the 27 Rab-binding proteins exhibited much broader Rab binding specificity than we had previously thought (Figs. 1, 2C, 3B, and 4A; e.g. OCRL and INPP5B each interacted with 16 distinct Rab isoforms). The physiological roles of their multiple Rab binding abilities in membrane trafficking remain largely unknown, but they may be involved in the redundancy or specificity of membrane trafficking. These Rab-binding proteins may regulate the same transport pathway (i.e. transport of the same cargo; redundant role) or different transport pathways (i.e. transport of different cargos; specific role) by binding to different Rab isoforms. Alternatively, these Rab-binding proteins may regulate different steps of one transport pathway by sequentially binding to different Rab isoforms as has been proposed in regard to endosomal trafficking by several endosomal Rabs (i.e. Rab conversion) (34). Further study is needed to address this issue.

We also identified 10 novel, specific Rab-binding proteins (Figs. 2C, 3B, and 4B), and some of them were further characterized by co-immunoprecipitation assay and immunofluorescence analysis (Figs. 2, D and E; 3, C and D; 4D; and 5, C and D). Two of them, GARI and GARI-L3, exhibited striking sequence similarity in their N-terminal domain, enabling us to identify a novel Rab2B-binding domain (RBD2B; ~200 amino acids) in combination with the standard protein BLAST search (Fig. 5, A and B). This domain is likely to be a Rab2B effector domain because it is necessary and sufficient for binding of Rab2B in a GTP-dependent manner (Fig. 5D) and for targeting to the Golgi where Rab2B is present (Fig. 5C). Another important finding is that RBD2B preferentially binds Rab2B but essentially does not bind Rab2A (Fig. 4B). This finding is also surprising because there is more than 85% amino acid identity between Rab2A and Rab2B, and members of the same subfamily of Rabs (e.g. Rab3A/B/C/D, Rab5A/B/C, or Rab27A/B) have been shown to function redundantly in certain membrane trafficking by previous studies (35–37). In view of this finding, we re-evaluated the Rab binding specificity of the 27 Rab-binding proteins identified in this study, and the results showed that three of them bind only the B isoform of Rabs and do not bind their A isoforms, although the majority of Rab-binding proteins bind both A and B isoforms. As an example, OCRL and INPP5B bind Rab22B but not Rab22A, and Golga4 binds Rab27B but not Rab27A (Table I). We therefore hypothesized that the functions of the A and B isoforms of Rabs in membrane trafficking are not always redundant and that the three Rab-binding proteins play unique roles through interaction with the B isoform of Rabs. Consistent with this hypothesis, distinct roles of Rab27A and Rab27B in mast cell granule dynamics (38) and of the Rab8A-specific effector (cenexin/ODF2) in cilium formation (40) have been reported while preparing this manuscript. Because we mainly used the B isoform of Rabs for screening, it would be interesting to screen for A isoform-specific effectors by using the A isoform of Rab as bait in the future.

The results of our large scale screening for novel Rab effectors also provided important information on the structure of Rab-binding domains. Approximately half of the Rab-binding proteins identified in this study contain CC domains, most of which have been shown to act as a Rab-binding domain (e.g. MICALs, MICAL-Ls, Cog4, Evi5, and Rab11-FIP2) (Refs. 25, 27, and 39 and this study). Future genome-wide investigations of the interaction between potential CC domains and Rabs are expected to reveal additional Rab-binding motifs.

Although two WD repeat-containing proteins, 4930573I19Rik and Wdr38, bound specific Rab isoforms (Fig. 4), WD repeats are generally unlikely to be a Rab-binding domain because the WD repeats of 4930573I19Rik are unnecessary for Rab binding activity (supplemental Fig. S1).

As far as we know, this is the first report of large scale screening for novel Rab effector molecules, and as a result of the screening we succeeded in identifying 19 novel Rab-binding proteins. We also determined their Rab binding specificity and found that most of the Rab-binding proteins have broader Rab binding specificity than we had thought, indicating the need to reassess the Rab binding specificity of previously described Rab effector molecules. Although the physiological significance of the multiple Rab binding ability of the Rab-binding proteins identified in this study remains unknown, the identification of 19 novel Rab-binding proteins and determination of their Rab binding specificity should greatly accelerate our understating of the molecular mechanism of Rab-mediated membrane trafficking.

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