Distinct Biochemical and Functional Properties of Two Rab5 Homologs from the Rice Blast Fungus *Magnaporthe oryzae*

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Background: It is unclear if the two Rab5 homologs in *M. oryzae* (MoRab5A and MoRab5B) are functional Rab5 orthologs. Results: Only MoRab5B resembles mammalian Rab5, while MoRab5A exhibits reduced GTPase and endocytosis activities. Conclusion: MoRab5B is the Rab5 ortholog. Significance: The study highlights that Rab5 homologs in fungi are not simply redundant Rab5 isoforms but represent distinct endosomal Rab functions.

Rab5 is a key regulator of early endocytosis by promoting early endosomal fusion and motility. In this study, we have unexpectedly found distinct properties of the two Rab5 homologs (MoRab5A and MoRab5B) from *Magnaporthe oryzae*, a pathogenic fungus in plants whose infection causes rice blast disease. Like mammalian Rab5, MoRab5A and MoRab5B can bind to several Rab5 effectors in a GTP-dependent manner, including EEA1, Rabenosyn-5, and Rabaptin-5. However, MoRab5A shows distinct binding characteristics in the sense that both the wild-type and the GTP hydrolysis-defective constitutively active mutant bind the effectors equally well in GST pull-down assays, suggesting that MoRab5A is defective in GTP hydrolysis and mostly in the GTP-bound conformation in the cell. Indeed, GTP hydrolysis assays indicate that MoRab5A GTPase activity is dramatically lower than MoRab5B and human Rab5 and is insensitive to RabGAP5 stimulation. We have further identified a Pro residue in the switch I region largely responsible for the distinct MoRab5A properties by characterization of MoRab5A and MoRab5B chimeras and mutagenesis. The differences between MoRab5A and MoRab5B extend to their functions in the cell. Although they both target to early endosomes, only MoRab5B closely resembles human Rab5 in promoting early endosome fusion and stimulating fluid phase endocytosis. In contrast, MoRab5A correlates with another related early endosomal Rab, Rab22, in terms of the presence of the switch I Pro residue and the blocked GTPase activity. Our data thus identify MoRab5B as the Rab5 ortholog and suggest that MoRab5A specializes to perform a non-redundant function in endosomal sorting.

Rab GTPases are molecular switches that regulate multiple aspects of vesicular transport in the intracellular membrane trafficking system of all eukaryotic cells (1–4). They are ancient proteins and evolutionarily conserved from the last eukaryotic common ancestor (LECA) to mammals. In LECA, there are at least 20 Rabs suggesting fundamental functions of the Rab proteins and intracellular membrane trafficking system in early eukaryotes (5, 6). During evolution, these Rab genes undergo both retraction and expansion depending on species. For example, there are less than a dozen Rab genes in fungi while there are nearly 70 Rab genes in mammals, reflecting the loss and expansion of organelles and membrane domains in cells. Only five Rabs appear indispensable throughout evolution including Rab1, Rab5, Rab6, Rab7, and Rab11, and they reflect the essential endocytic and exocytic functions in early eukaryotes (5, 6). These Rabs have multiple homologs, which poses a challenge for identification of authentic orthologs between species.

Rab5 in particular has three isoforms in mammalian cells (7), and there are three Rab5 homologs in the budding yeast *Saccharomyces cerevisiae* (*S. cerevisiae*) (8). However, it’s still unclear how each yeast homolog correlates evolutionarily and functionally with the Rab5 isoforms in mammals. In addition, there are several Rabs closely related to Rab5 such as Rab17 (9), Rab21 (10), and Rab22 (11–14) that co-localize with Rab5 on early endosomes in mammalian cells, and they are not found in yeast and other fungi by phylogenetic analysis. However, phylogenetic information alone cannot rule out the possibility that one of the currently annotated Rab5 homologs in lower eukaryotes such as fungi may represent a functional counterpart for one of the early endosomal Rab5 subfamily members rather than Rab5 itself in mammalian cells.

To address this issue, we have cloned the two Rab5 homologs (MGG_06241 and MGG_01185 termed here as MoRab5A and MoRab5B) from *Magnaporthe oryzae* (*M. oryzae*), which is a pathogenic fungus in plants and causes rice blast disease (15), and characterized their biochemical properties and determined if they exhibit the same function in stimulating endocytosis in

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mammalian cells. Rab5 normally targets to early endosomes and promotes early endosome fusion and endocytosis (16–20). We find that both MoRab5A and MoRab5B localize to early endosomes but only MoRab5B potently stimulates endocytosis in mammalian cells like human Rab5 (hRab5). In addition, it is well documented that constitutively active (CA) GT-Pase-defective mutants of Rab5 (e.g. the Gln79 to Leu mutation in hRab5) (19, 20) dramatically increase the size of early endosomes in cells by promoting endosome fusion. In this regard, MoRab5B also closely resembles hRab5, but not MoRab5A. Taken together with their interaction profiles with a panel of effectors including Rabaptin-5 (21), Rabenosyn-5 (22), and EEA1 (23), we conclude that MoRab5B represents the authentic ortholog of mammalian Rab5 while MoRab5A plays a distinct specialized function, possibly in endosomal sorting.

EXPERIMENTAL PROCEDURES

Plasmids and cDNAs—The cDNAs of MoRab5A (MGG_06241) and MoRab5B (MGG_01185) were isolated by RT-PCR of total RNAs from the mycelium culture of M. oryzae strain Guy11 and cloned into the pGEM-T Easy vector (Promega), followed by verification with direct DNA sequencing. For expression in mammalian cells, the MoRab5A and MoRab5B cDNAs were amplified by PCR with oligonucleotide primers encoding an N-terminal Myc epitope and cloned into the MLul/Nhel sites of the tetracycline-regulatable pBI or pBI/eGFP expression vector (Clontech Laboratories, Inc.). pTet-Off and pTet-On vectors were also obtained from Clontech Laboratories, Inc.

CA, dominant negative (DN), and other mutants were generated by site-directed mutagenesis using the Quick-Change kit (Agilent Technologies), leading to mutant cDNAs MoRab5A: Q70L (CA), MoRab5A:S25N (DN), MoRab5A:P42S, MoRab5B: Q79L (CA), MoRab5B:S33N (DN), and MoRab5B:S50P, which were then cloned into pBII, pBI/eGFP, or pLenti-CMV-IRESeGFP-PGK-Puro vector. The mutations were verified by direct DNA sequencing. Human Rab5 counterparts including wild-type (WT) and mutants (hRab5:Q79L and hRab5:S34N) in pBI or pBI/eGFP vector were described previously (14, 24), and were also cloned into pLenti-CMV-IRESeGFP-PGK-Puro vector for production of recombinant lentiviruses. The hRab5 used in this study refers to hRab5a.

For expression in bacteria, the cDNAs of hRab5, MoRab5A, and MoRab5B were cloned into the pGEX-4T-2 vector (GE Healthcare) and the resulting pGEX-4T-2-hRab5, pGEX-4T-2/MoRab5A and pGEX-4T-2/MoRab5B constructs were transformed into the Escherichia coli strain DH5α for expression of GST-hRab5, GST-MoRab5A, and GST-MoRab5B fusion proteins.

Antibodies—Anti-actin and anti-Myc monoclonal antibodies (mAb) were purchased from Sigma-Aldrich, whereas the anti-Rab5 mAb was from BD Biosciences.

Cell Culture and Transfection—Baby hamster kidney (BHK)-21 cell monolayers were grown in 35-mm culture dishes with 2 ml of α-minimal essential medium (MEM) containing 5% heat-inactivated fetal bovine serum (FBS) and 20 units/ml penicillin/streptomycin (Invitrogen), while Tet-Off PC12 cells (Clontech Laboratories, Inc.) were grown in poly-lysine coated 35-mm culture dishes with 2 ml of Dulbecco-modified MEM (DMEM) containing 10% heat-inactivated horse serum, 5% heat-inactivated FBS, 20 units/ml penicillin/streptomycin, 1 mM l-glutamine, and 200 μg/ml Geneticin (G-418; Invitrogen). Cells were maintained in humidified 37 °C incubators with 5 and 10% CO2 for BHK and PC12 cells, respectively. For protein expression, cell monolayers were transfected with the indicated plasmid constructs via the Lipofectamine 2000-mediated procedure (Invitrogen), and incubated at 37 °C for 24 h. Cell lysates were either directly analyzed for protein expression by immunoblot assay with anti-Myc and anti-Rab5 antibodies or used for glutathione S-transferase (GST) pull-down assays. For intracellular localization, cells were fixed by 4% paraformaldehyde and observed by confocal fluorescence microscopy as described below.

Confocal Fluorescence Microscopy—A Leica SP2 MP confocal laser scanning microscope with six laser excitation including 488, 543, and 594 nm in the Flow and Image Cytometry Laboratory (University of Oklahoma Health Sciences Center) was used by following a procedure described previously (14). Briefly, BHK-21 or PC12 cells were grown on cover-slips and transfected with the pBI constructs expressing eGFP (enhanced green fluorescent protein) tagged MoRab5A, MoRab5B, and tdTomato tagged hRab5 fusion proteins as indicated. The pBI vector is a bi-directional expression vector and can express an eGFP fusion protein and a tdTomato fusion protein simultaneously. At 24 h post-transfection, the cells were rinsed three times in PBS and fixed for 15 min with 4% paraformaldehyde (v/v in PBS) in a 37 °C Incubator. The cover-slips were then mounted on glass slides in ProLong® Gold or SlowFade® Gold Antifade Reagents and viewed with the confocal microscope.

GST Pull-down Assay—The Rab5-binding domains (R5BD) of Rabaptin-5, Rabenosyn-5, and EEA1 were expressed as GST fusion proteins in the E. coli strain DH5α transformed with the pGEX constructs pGEX-4T-2/Rabaptin-5:R5BD (24), pGEX-2T/Rabenosyn-5:R5BD (25) (kindly provided by John Colicelli of UCLA) and pGEX-4T-2/EEA1:R5BD (24), respectively. Protein expression was induced by addition of 1 mM isopropyl-β-D-thiogalactoside (IPTG) for 4 h at 37 °C. GST-R5BD fusion proteins were then affinity-purified with glutathione-Sepharose 4B resin (GE Healthcare) by following the manufacturer’s instructions. GST alone was expressed from the empty pGEX vector and purified as a negative control. A fraction of each purified GST fusion protein (5%) was analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) (12% gel) and visualized by Coomassie Brilliant Blue staining. Another fraction was used for quantification of protein concentration by the Bio-Rad protein assay. The remainder was used for pull-down assay by incubation with the cell lysates from BHK cell monolayers transfected with the pBI constructs expressing Myc-tagged hRab5, MoRab5A, MoRab5B, and mutants, respectively, using a procedure described previously (14).

GTP Hydrolysis Assay—GST-hRab5, GST-MoRab5A, and GST-MoRab5B fusion proteins were affinity-purified in the same way as the GST-R5BD fusion proteins described above and 1 μM of each bound to glutathione-Sepharose 4B resin were incubated with [α-32P]GTP (0.1 μM) (Amersham Biosciences) for 15 min with occasional rocking at room temperature in...
50 μl of loading buffer (20 mM Tris-HCl, pH 8.0, 5 mM EDTA, 1 mM dithiothreitol (DTT)). GTP hydrolysis reaction was initiated by resuspending the resin in reaction buffer (20 mM Tris-HCl, pH 8.0, 10 mM MgCl₂, 1 mM DTT), and incubating at 30 °C in the absence or presence of 10 nM RabGAP5. Samples were taken at the indicated times and immediately solubilized in elution buffer (20 mM Tris-HCl, pH 8.0, 2% (w/v) SDS, 5 mM EDTA, 5 mM GDP, 5 mM GTP) by heating at 70 °C for 2 min. The eluted GTP and GDP were separated by thin layer chromatography (TLC) on polyethyleneimine-cellulose sheets (JT Baker) with 0.75 M NaH₂PO₄, pH 3.4, as the developing solvent, followed by visualization by autoradiography and quantification by PhosphorImager (Molecular Dynamics).

Subcellular Fractionation—BHK cell monolayers were transfected with the pBLeGFP constructs expressing Myc-tagged hRab5, MoRab5A, MoRab5B, and the indicated mutants, and incubated at 37 °C for 24 h. Then the cells were rinsed with ice-cold PBS and homogenized in 250 μl of TE buffer (100 mM Tris-HCl, pH 7.4, 1 mM EDTA) by passing through a 1-ml syringe with 25G/8 needle 10 times. Cell lysates were centrifuged at 800 × g for 3 min, the supernatants were then subjected to ultra-speed centrifugation at 200,000 × g for 7 min in a Beckman-Coulter MAX-XP ultracentrifuge to separate the membrane fraction (pellet) from the cytosol fraction (supernatant). The membrane pellet was resuspended in the same volume of TE buffer as the cytosol fraction (supernatant). The membrane fraction was added to 20% (v/v) Triton X-100 for 2 h. After clarification of cell debris by centrifugation at 200,000 × g for 5 min to remove nuclei and cell debris, and post-nuclear supernatants were then subjected to ultra-speed centrifugation at 200,000 × g for 7 min in a Beckman-Coulter MAX-XP ultracentrifuge to separate the membrane fraction (pellet) from the cytosol fraction (supernatant). The membrane pellet was resuspended in the same volume of TE buffer as the cytosol fraction, and SDS (from 10% stock) was added to both fractions for a final concentration of 1%. The hRab5, MoRab5A, and MoRab5B proteins in each fraction (20 μl) were analyzed by SDS-PAGE (12% gel) and immunoblot assay.

Horseradish Peroxidase (HRP) Uptake Assay—BHK cells were infected with recombinant lentiviruses expressing Myc-tagged hRab5, MoRab5A, MoRab5B, and their DN mutants (hRab5:S34N, MoRab5A:S25N, and MoRab5B:S33N), respectively, and selected by 1 μg/ml puromycin (BD Biosciences) until all cells were transduced as evidenced by the expression of eGFP marker. In addition, a second set of cells were transiently transfected with the pBI/eGFP constructs expressing the Myc-tagged hRab5, MoRab5A, MoRab5B, and their DN mutants (hRab5:S34N, MoRab5A:S25N, and MoRab5B:S33N), respectively, and selected by 1 μg/ml puromycin (Invitrogen) in the presence of 8 μg/ml of polybrene (Sigma-Aldrich), and selected in the presence of 1 μg/ml puromycin (Invitrogen).

RESULTS

Molecular Cloning of MoRab5A and MoRab5B from M. oryzae—Based on the M. oryzae genome database (previously called *Magnaporthe grisea*), we identified and cloned two Rab5 homologs MGG_06241 and MGG_01185 and termed them MoRab5A and MoRab5B, respectively. Sequence alignment with hRab5 indicated five conserved GTP-binding motifs (G1–5) and Rab5-like switch I and II regions in both MoRab5A and MoRab5B (Fig. 1A), suggesting that they may interact with common effectors. MoRab5B exhibited more amino acid sequence homology with hRab5 than MoRab5A did, with 63% and 58% identity, respectively. In addition, MoRab5A contained a large insert (20 amino acids) between G4 and G5 motifs and a small one in the C-terminal hypervariable region (Fig. 1A). To determine if MoRab5A or MoRab5B may represent a functional ortholog of mammalian Rab5, we compared the biochemical properties of MoRab5A and MoRab5B with hRab5 and investigated if MoRab5A and MoRab5B can mimic hRab5 functions in mammalian cells. In the following biochemical and functional assays, the three hRab5 isoforms showed the same activities (7) and hRab5A was used in the experiments described below.

MoRab5A and MoRab5B Interact with Rab5 Effectors Rabaptin-5, Rabenosyn-5, and EEA1 but Show Distinct Binding Characteristics—Upon GTP binding and activation, Rab5 binds to a number of effector proteins to promote early endosome fusion and endocytosis. In mammalian cells, some of these Rab5 effectors are well characterized, such as Rabaptin-5 (21), Rabenosyn-5 (22) and EEA1 (23). We tested whether the two fungal Rab5 homologs, MoRab5A and MoRab5B, can be...
expressed in BHK cells and interact with the three Rab5 effectors in GST-R5BD pull-down assays where R5BD refers to the Rab5-binding domain from Rabaptin-5, Rabenosyn-5, and EEA1, respectively. Like hRab5, both MoRab5A and MoRab5B as well as their CA and DN mutants were robustly expressed via the pBI/eGFP expression vector (Fig. 1B, Input). MoRab5B exhibited doublet bands on the gel, likely due to a higher expression level and saturation of the Rab geranylgeranyltransferase in the cell, leading to accumulation of the unprenylated, slower mobility precursor form (29). Importantly, the MoRab5A and MoRab5B proteins appeared properly folded and exhibited Rab5 characteristics in terms of GTP-dependent interaction with the three Rab5 effectors (Fig. 1B, pull-down). MoRab5B was similar to hRab5 in size (25–26 kDa) and showed the same effector interaction characteristics as hRab5, i.e. the GTPase-defective GTP-bound CA mutant showed the strongest binding signal, WT binding was much reduced while the GDP-bound DN mutant showed little binding similar to the background by GST alone (Fig. 1B, pull-down). In contrast, MoRab5A was larger in size (34–35 kDa) and showed a distinct interaction profile where WT was the same as the CA mutant in terms of binding to the three effectors (Fig. 1B, pull-down), suggesting that MoRab5A may not be able to hydrolyze GTP efficiently in the cell and is locked in the GTP-bound conformation. In support of this contention, for the WT proteins MoRab5A showed stronger effector binding than MoRab5B and hRab5 (Fig. 1B, pull-down).

MoRab5A Exhibits Low GTPase Activity—Next, we directly determined intrinsic and GAP-accelerated GTPase activity by MoRab5A and MoRab5B, in comparison to hRab5. Recombinant proteins GST-MoRab5A, GST-MoRab5B and GST-hRab5 were expressed in E. coli and affinity-purified with glutathione-Sepharose resin, followed by loading with $[^{32}P]GTP$ and GTP hydrolysis reaction at 30 °C in the absence and presence of RabGAP5 (30). Samples were taken after 0, 10, and 20 min, and GDP and GTP were separated and visualized by TLC and autoradiography (Fig. 2). Like hRab5, MoRab5B exhibited relatively fast intrinsic GTP hydrolysis rate, which was further accelerated by RabGAP5 (30) with nearly complete conversion from GTP to GDP in 20 min (Fig. 2A). In contrast, MoRab5A showed dramatically lower intrinsic GTPase activity and little sensitivity to RabGAP5 stimulation with less than 20% GTP to GDP conversion (Fig. 2A), even though MoRab5A contains the same five conserved GTP-binding motifs (G1–5, Fig. 1A).

A Pro Residue in the Switch I Region Is Responsible for the Distinct Effector-binding Property and GTPase Activity of MoRab5A—To determine if there is a domain or motif in
MoRab5A that may distinguish it from MoRab5B in terms of effector binding profile and GTPase activity described above, we made a series of MoRab5A and MoRab5B chimeras and expressed them as Myc-tagged proteins in BHK cells, followed by GST-R5BD pull-down assays. The initial six chimeras tested were 5A-I/B, 5A-III/B, 5A-IV/B, 5B-I/A, 5B-III/A, and 5B-IV/A, with the first three containing N-terminal MoRab5A fragments up to the first, the third and the fourth GTP-binding motif (G1, G3, and G4) fused in-frame to corresponding C-terminal MoRab5B fragments, and the last three representing reciprocal chimeras containing N-terminal MoRab5B and C-terminal MoRab5A fragments (Fig. 3A). With MoRab5A and MoRab5B as controls for strong and weak effector binding (Fig. 3, see also Fig. 1B), respectively, in the GST-R5BD pull-down assay, we found that 5A-I/B exhibited weak effector binding like MoRab5B while 5A-III/B and 5A-IV/B exhibited strong binding like MoRab5A, suggesting that a region between G1 and G3 is responsible for the difference between MoRab5A and MoRab5B (Fig. 3B). This contention was corroborated by results on the reciprocal chimeras, with 5B-I/A mimicking MoRab5A and 5B-III/A and 5B-IV/A mimicking MoRab5B in terms of effector binding strength (Fig. 3C).
We further narrowed down the effector-binding determinant by making and testing additional chimeras around the second GTP-binding motif (G2), i.e., the conserved Thr residue in the switch I region, including 5A-II\textsubscript{36}/B, 5A-II\textsubscript{42}/B, 5A-II\textsubscript{55}/B, 5B-II\textsubscript{44}/A, 5B-II\textsubscript{50}/A, and 5B-II\textsubscript{63}/A (Fig. 3A). The 5A-II\textsubscript{36}/B construct contained an N-terminal MoRab5A fragment up to residue 36 (a conserved Phe) and the corresponding C-terminal MoRab5B fragment, and it exhibited the same weak effector binding as MoRab5B (Fig. 3B). In contrast, 5A-II\textsubscript{42}/B and 5A-II\textsubscript{55}/B exhibited the strong effector binding property of MoRab5A (Fig. 3B). Taken together, the data suggested that the strong effector-binding determinant of MoRab5A resides between residues 36 and 42. This contention was corroborated by the reciprocal chimeras with N-terminal MoRab5B and C-terminal MoRab5A fragments (5B-II\textsubscript{44}/A, 5B-II\textsubscript{50}/A, and 5B-II\textsubscript{63}/A) (Fig. 3A). In this regard, 5B-II\textsubscript{44}/A resembled MoRab5A while 5B-II\textsubscript{63}/A and 5B-II\textsubscript{50}/A resembled MoRab5B in terms of effector binding strength (Fig. 3C).

Sequence alignment of residues 36 – 42 of MoRab5A with the corresponding regions of MoRab5B and hRab5 led us to identify a candidate position immediately upstream of the conserved G2 Thr residue that may distinguish MoRab5A from MoRab5B and hRab5, a Pro in the former but a Ser in the latter proteins (Fig. 3A). Thus we made a substitution mutant replacing Pro with Ser at residue 42 in MoRab5A (MoRab5A:P42S) and the reciprocal MoRab5B mutant (MoRab5B:S50P) and...
determined if the mutations can switch the effector-binding property and GTPase activity of MoRab5A and MoRab5B in the GST-R5BD pull-down and GTP hydrolysis assays. Indeed, MoRab5A:P42S showed reduced effector binding like MoRab5B (Fig. 3B) while MoRab5B:S50P showed enhanced effector binding like MoRab5A (Fig. 3C). Consistently, MoRab5A:P42S showed enhanced intrinsic GTPase activity, which became sensitive to RabGAP5 stimulation like MoRab5B, while MoRab5B:S50P showed reduced intrinsic GTPase activity, which was no longer sensitive to RabGAP5 stimulation like MoRab5A (Fig. 2B). The position of Pro42 in MoRab5A is immediately upstream of the conserved G2 Thr residue (Fig. 3A), which normally coordinates Mg$^{2+}$ in GTP binding and hydrolysis. Our data suggest that Pro$^{42}$ may hinder the G2 Thr function and thereby block the GTPase activity of MoRab5A, leading to increased levels of active GTP-bound form for effector binding.

MoRab5A and MoRab5B Target to Early Endosomes and Co-localize with hRab5 in the Cell but Only MoRab5B Promotes Early Endosome Fusion—To determine if MoRab5A and MoRab5B can target to early endosomes like hRab5, we expressed them as GFP fusion proteins in BHK cells and observed their intracellular localization via confocal fluorescence microscopy. Like hRab5, both MoRab5A and MoRab5B targeted to punctate structures in the cell (Fig. 4A), which were early endosomes rather than late endosomes as evidenced by their co-localization with hRab5 but not hRab7 (Fig. 4, B–D). However, only MoRab5B exhibited endosome fusion activity, like hRab5, by the enlargement of early endosomes in cells expressing the constitutively active CA mutants (Fig. 4A). The MoRab5B:S50P mutant with reduced GTPase activity (Fig. 2) also enlarged the endosomes despite to a lesser degree (Fig. 4A). In contrast, MoRab5A:WT- and MoRab5A:CA-labeled endosomes were similar and they were smaller and rarely enlarged, although they were often clustered (Fig. 4A). In this regard, the MoRab5A:PS mutation, with increased GTPase activity (Fig. 2) also exhibited similar endosomal morphology (Fig. 4A). The experiment was also done in another cell type, PC12 cells, and the results were the same (data not shown).

We further determined the steady-state membrane and cytosolic distribution of MoRab5A and MoRab5B in the cell by subcellular fractionation. In this regard, Myc-tagged hRab5, MoRab5A, MoRab5B and their CA and DN mutants were expressed in BHK cells, and the cells were homogenized and post-nuclear supernatants were subjected to ultracentrifugation to separate membrane and cytosol fractions. The Rab5 proteins in each fraction were identified by immunoblot analysis with an anti-Myc antibody (Fig. 4E). Like hRab5, MoRab5B were about equally distributed in the membrane and...
cytosol with small increase and decrease in membrane association by the CA and DN mutants, respectively (Fig. 4E). In comparison, MoRab5A and mutants showed slightly less membrane association (Fig. 4E).

The membrane association of Rab GTPases generally reflects their post-translational isoprenylation and interactions with GEFs (31) and/or GDFs (32) while the cytosolic fraction reflects their retrieval by GDI as well as a newly synthesized precursor pool. Since the mostly GTP-bound MoRab5A shows no more membrane association than MoRab5B and hRab5, our data suggest that membrane targeting rather than retrieval by GDI is rate-limiting in the cell. Indeed, in the case of MoRab5B where the unprenylated precursor form and the prenylated mature form are clearly separated by SDS-PAGE, the cytosolic fraction contains mostly the slower mobility precursor form (Fig. 4E), suggesting that the Rab prenyltransferase activity is limiting in the cell and cannot convert all MoRab5B molecules to the mature form for membrane association. Along this line, the MoRab5A results can be reconciled, with the membrane and cytosol fractions reflecting the mature and precursor forms, respectively, even though the two forms are not readily resolved on the gel (Fig. 4E). The smaller membrane fraction of MoRab5A suggests lower efficiency of interaction with the Rab prenyltransferase and/or GEF/GDF for membrane association.

MoRab5B, Like hRab5, Is a Robust Stimulator of Endocytosis while MoRab5A Shows Little Effect—We further tested if MoRab5A and MoRab5B can stimulate cellular endocytic activity upon overexpression in BHK cells, like hRab5 (17, 19). To this end, we made recombinant lentiviruses expressing Myc-tagged hRab5, MoRab5A, MoRab5B and their dominant negative DN mutants, respectively, and used them to infect BHK cells, followed by selection of infected cells by puromycin to homogeneity, as evidenced by expression of the GFP marker from the lentiviral vector in all cells. Each protein expression was confirmed by immunoblot analysis with an anti-Myc antibody, and the levels of hRab5 and MoRab5A proteins were similar but somewhat lower than those of MoRab5B (Fig. 5A). The actin level in each sample served as a loading control (Fig. 5A). Endocytic activity of each cell line was determined by HRP uptake for 1 h. In comparison to the control cells (vector), MoRab5B strongly stimulated HRP uptake, like hRab5 (Fig. 5B). In contrast, MoRab5A showed little stimulatory effect (Fig. 5B), even though its expression level was similar to that of hRab5 (Fig. 5A). None of the DN mutants showed any stimulatory effect, neither were they effective inhibitors (Fig. 5B). Previous studies suggest that high levels of expression may be necessary for Rab5 DN mutants to block endocytosis via sequestration of GEFs (17, 19).

We further substantiated the results by transient expression of another set of Rab5 proteins including hRab5:WT, hRab5:CA, MoRab5A:WT, MoRab5A:P42S, MoRab5B:WT, and MoRab5B:S50P (Fig. 5C), and tested their effect on HRP uptake (Fig. 5D). Consistent with the stable expression above (Fig. 5B), transient overexpression of hRab5:WT or MoRab5B:WT strongly stimulated HRP uptake while MoRab5A:WT showed little stimulatory effect (Fig. 5D). Like hRab5:WT, the constitutively active hRab5:CA mutant stimulated HRP uptake. In this regard, the MoRab5B:S50P mutant resembled the constitutively active mutant with reduced GTPase activity and increased level of active GTP-bound form (Fig. 2), and also stimulated HRP uptake (Fig. 5D). The MoRab5A:P42S mutant, on the other hand, did not stimulate HRP uptake, like MoRab5A:WT (Fig. 5D).

Taken together with the data in Fig. 4A, we conclude that MoRab5B but not MoRab5A functions as a Rab5 ortholog in terms of stimulation of endosome fusion and endocytosis. While the Pro and Ser switch in the switch I region may contribute to the difference in GTPase activity between MoRab5A and MoRab5B, it cannot account for their functional differences, suggesting that MoRab5A may represent a distinct endosomal sorting function, similar to another member of the Rab5 subfamily in mammalian cells, Rab22, which also contains a Pro residue at the equivalent switch I position and exhibits low GTPase activity.

HRP Is Endocytosed into Rab5-positive Early Endosomes—HRP is a well-documented fluid phase endocytic marker (26) but we wanted to confirm that it indeed enters the Rab5-positive early endosomes. The early endosomes were marked in BHK cells by expression of GFP-hRab5, GFP-MoRab5A, and GFP-MoRab5B, respectively, and HRP was endocytosed for 20 min, followed by fixation and staining of HRP with its substrate DAB. The confocal fluorescent images of Rab5-positive early endosomes were superimposed onto the phase-contrast images of HRP-positive structures (Fig. 6). HRP was found inside Rab5-positive early endosomes. The early endosomes were marked in BHK cells by expression of GFP-hRab5, GFP-MoRab5A, and GFP-MoRab5B, respectively, and HRP was endocytosed for 20 min, followed by fixation and staining of HRP with its substrate DAB. The confocal fluorescent images of Rab5-positive early endosomes were superimposed onto the phase-contrast images of HRP-positive structures (Fig. 6). HRP was found inside Rab5-positive early endosomes, including GFP-hRab5 (Fig. 6A), GFP-MoRab5A (Fig. 6B), and GFP-MoRab5B (Fig. 6C) labeled structures, respectively. The results are consistent with our conclusion that both MoRab5A and MoRab5B co-localize with hRab5 to early endosomes (Fig. 4), even though MoRab5A does not significantly enhance endosome fusion and endocytosis activity, unlike MoRab5B and hRab5 (Fig. 5).

DISCUSSION

We have identified and characterized two Rab5 homologs (MoRab5A and MoRab5B) from the rice blast fungus M. oryzae and found unexpectedly that only MoRab5B can mimic hRab5 function in promoting early endosome fusion and endocytosis in mammalian cells and exhibit the same biochemical characteristics as hRab5, indicating that it is an authentic Rab5 ortholog. In this regard, MoRab5B targets to endosomes and co-localizes with hRab5. Importantly, overexpression of MoRab5B, especially the GTP-bound CA mutant, dramatically enlarges early endosomes in the cell like hRab5, indicating enhanced early endosome fusion. Furthermore, overexpression of MoRab5B potently stimulates fluid phase endocytosis (HRP uptake...
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uptake), a hallmark of Rab5 function in mammalian cells (17, 19). MoRab5A also resembles hRab5 in intrinsic and RabGAP5-accelerated GTPase activities.

In contrast, MoRab5A shows much lower intrinsic GTPase activity and little sensitivity to RabGAP5 stimulation. In addition, MoRab5A shows little endosome fusion activity and enlargement of early endosomes in the cell, although it can target to Rab5-positive early endosomes and interact with the Rab5 effectors tested including Rabaptin-5, Rabenosyn-5, and EEA1. A closer look at the confocal fluorescence microscopy images reveals larger, aggregated MoRab5A- and MoRab5A:CA-associated structures, possibly due to endosomal tethering without fusion to form enlarged vesicles, which is consistent with the tethering function of Rabenosyn-5 and EEA1 (33) and suggests additional Rab5 effectors necessary for membrane fusion that MoRab5A cannot effectively recruit in the cell, e.g. the CORVET complex (34). It is also possible that MoRab5A may have a higher affinity for and preferentially bind an effector to promote a function other than endosome fusion, e.g. endosome formation or movement in the cell, in comparison to hRab5 and MoRab5B.

In mammals, the three Rab5 isoforms are grouped together with several evolutionarily related Rabs such as Rab17, Rab20, Rab21, Rab22, and Rab24 based on phylogenetic analysis (5, 6, 35). Among them, Rab22 is most closely related to and as ancient as Rab5 in the sense that it exists in LECA. In this regard, Rab22 also interacts with several Rab5 effectors such as EEA1 and Rabenosyn-5 (11, 36) but it does not promote the degradative endocytic pathway from early to late endosomes. Instead, Rab22 regulates early endosomal sorting and recycling (11–13) and neurite outgrowth (37). However, Rab22 appears lost in fungi including yeast and M. oryzae discussed here (5, 6, 33). We speculate that one of the fungal Rab5 homologs, MoRab5A, may compensate for the loss of Rab22 and specialize to perform a Rab22-like endosomal function, although such a function is not yet clearly defined in M. oryzae. In PC12 cells, MoRab5A cannot mimic Rab22 in promoting neurite outgrowth, but it does show the same low GTPase activity and effector binding characteristics as Rab22, i.e. WT is mostly GTP-bound at steady state and no different from the CA mutant in terms of binding Rab5 effectors Rabenosyn-5 and EEA1 in GST pull-down assays (data not shown). We have mapped the signature motif responsible for such characteristics to be a single Pro residue immediately upstream of the conserved G2 residue Thr in the switch I region. Interestingly, Rab22 is only one of 3 mammalian Rabs with a Pro residue at this position excluding isoforms while Ser/Thr or Ala residue is prevalent at the corresponding position. In the budding yeast S. cerevisiae, one of the three Rab5 homologs, Ypt51/Vps21, also contains a Pro residue at the corresponding position (8) and is more homologous to MoRab5A than MoRab5B, suggesting that it may have similar reduced GTPase activity in comparison to Ypt52, which is more homologous to MoRab5B.

Fluid-phase endocytosis is active in the conidia cells of M. oryzae and is suggested to play an important role in the fungal development and infection of plants (38). Further understanding of the biological functions of endocytosis, specifically MoRab5A- and MoRab5B-mediated early endosomal processes, may involve knocking out the MoRab5A and MoRab5B genes or knocking down their expression via RNAi in M. oryzae and investigating the effects on M. oryzae development, such as sporulation, appressorial formation, and pathogenicity in rice.

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REFERENCES

1. Li, G., and Segev, N. (2012) Ypt/Rab GTPases and Intracellular Membrane Trafficking: An Overview in Rab GTPases and Membrane Trafficking (Li, G., and Segev, N., eds) pp. 3–17, Bentham Science Publishers
2. Mizuno-Yamasaki, E., Rivera-Molina, F., and Novick, P. (2012) GTPase networks in membrane traffic. Annu. Rev. Biochem. 81, 637–659
3. Pfeffer, S. R. (2013) Rab GTPase regulation of membrane identity. Curr. Opin. Cell Biol. 25, 414–419
4. Stenmark, H. (2009) Rab GTPases as coordinators of vesicle traffic. Nat. Rev. Mol. Cell Biol. 10, 513–525
5. Diekmann, Y., Seixas, E., Gouw, M., Tavares-Cadete, F., Seabra, M. C., and Gallwitz, D., and Zerial, M. (1995) (1994) Role of three rab5-like GTPases, Ypt1p, Ypt52p, and Ypt53p, in the endocytic and vacuolar protein sorting pathways of yeast. J. Cell Biol. 125, 283–298
6. Zacchi, P., Stenmark, H., Parton, R. G., Orioli, D., Lim, F., Giner, A., Mell, L., Zerial, M., and Murphy, C. (1998) Rab17 regulates membrane trafficking through apical recycling endosomes in polarized epithelial cells. J. Cell Biol. 140, 1039–1053
7. Simpson, J. C., Griffiths, G., Wessling-Resnick, M., Fransen, J. A., Bennett, H., and Jones, A. T. (2004) A role for the small GTPase Rab22 in the early endocytic pathway. J. Cell Sci. 117, 6297–6311
8. Kauppi, M., Simonsen, A., Bremnes, B., Vieira, A., Callaghan, J., Stenmark, H., and Oikonen, V. M. (2002) The small GTPase Rab22 interacts with EEA1 and controls endosomal membrane trafficking. J. Cell Sci. 115, 899–911
9. Magadán, J. G., Barbieri, M. A., Mesa, R., Stahl, P. D., and Mayorga, L. S. (2006) Rab22 regulates the sorting of transferrin to recycling endosomes. Mol. Biol. Cell 26, 2595–2614
10. Weigert, R., Yeung, A. C., Li, J., and Donaldson, J. G. (2004) Rab22a regulates the recycling of membrane proteins internalized independently of clathrin. Mol. Biol. Cell 15, 3758–3770
11. Zhu, H., Liang, Z., and Li, G. (2009) Rabex-5 is a Rab22 effector and mediates a Rab22-Rab5 signaling cascade in endocytosis. Mol. Biol. Cell 20, 4720–4729
12. Ebbole, D. J. (2007) Magnaporthe as a model for understanding host-pathogen interactions. Annu. Rev. Phytopathol. 45, 437–456
13. Barbieri, M. A., Li, G., Colombo, M. I., and Stahl, P. D. (1994) Rab5, an early acting endosomal GTPase, supports in vitro endosome fusion without GTP hydrolysis. J. Biol. Chem. 269, 18720–18722
14. Bucci, C., Parton, R. G., Mather, I. H., Stunnenberg, H., Simons, K., Hock, B., and Zerial, M. (1992) The small GTPase rab5 functions as a regulatory factor in the early endocytic pathway. J. Cell Sci. 103, 6297–6311
15. Li, G., and Stahl, P. D. (1993) Structure-function relationship of the small GTPase rab5. J. Biol. Chem. 268, 24475–24480
16. Stenmark, H., Parton, R. G., Steele-Mortimer, O., Lüttke, A., Gruenberg, J. Cell Biol. 140, 1039–1053
17. Gutierrez, J. P., Chavrier, P., Zerial, M., and Gruenberg, J. (1991) rab5 co-operative regulation of endocytosis by three Rab5 isoforms. FEBS Lett. 366, 65–71
18. Diekmann, Y., Seixas, E., Gouw, M., Tavares-Cadete, F., Seabra, M. C., and Zerial, M. (1995) The small GTPase Rab22 interacts with EEA1 in GST pull-down assays (data not shown). We have mapped the signature motif responsible for such characteristics to be a single Pro residue immediately upstream of the conserved G2 residue Thr in the switch I region. Interestingly, Rab22 is only one of 3 mammalian Rabs with a Pro residue at this position excluding isoforms while Ser/Thr or Ala residue is prevalent at the corresponding position. In the budding yeast S. cerevisiae, one of the three Rab5 homologs, Ypt51/Vps21, also contains a Pro residue at the corresponding position (8) and is more homologous to MoRab5A than MoRab5B, suggesting that it may have similar reduced GTPase activity in comparison to Ypt52, which is more homologous to MoRab5B.
Inhibition of rab5 GTPase activity stimulates membrane fusion in endocytosis. EMBO J. 13, 1287–1296

Stenmark, H., Vitale, G., Ullrich, O., and Zerial, M. (1995) Rabaptin-5 is a direct effector of the small GTPase Rab5 in endocytic membrane fusion. Cell 83, 423–432

Nielsen, E., Christoforidis, S., Uttenweiler-Joseph, S., Miaczynska, M., Dewitte, F., Wilm, M., Hoflack, B., and Zerial, M. (2000) Rabenosyn-5, a novel Rab5 effector, is complexed with hVPS45 and recruited to endosomes through a FYVE finger domain. J. Cell Biol. 151, 601–612

Simonsen, A., Lippé, R., Christoforidis, S., Gaullier, J. M., Brech, A., Callaghan, J., Toh, B. H., Murphy, C., Zerial, M., and Stenmark, H. (1998) EEA1 links PI(3)K function to Rab5 regulation of endosome fusion. Nature 394, 494–498

Liu, J., Lamb, D., Chou, M. M., Liu, Y. J., and Li, G. (2007) Nerve growth factor-mediated neurite outgrowth via regulation of Rab5. Mol. Biol. Cell 18, 1375–1384

Balaji, K., Mooser, C., Janson, C. M., Bliss, J. M., Hojjat, H., and Colicelli, J. (2012) RIN1 orchestrates the activation of RAB5 GTPases and ABL tyrosine kinases to determine the fate of EGFR. J. Cell Sci. 125, 5887–5896

Steinman, R. M., Silver, J. M., and Cohn, Z. A. (1974) Pinocytosis in fibroblasts. Quantitative studies in vitro. J. Cell Biol. 63, 949–969

Berry, W. L., Kim, T. D., and Janknecht, R. (2014) Stimulation of beta-catenin and colon cancer cell growth by the KDM4B histone demethylase. Int. J. Oncol. 44, 1341–1348

Marino, M., Luce, M., and Reiser, J. (2003) Small-to-large scale production of lentivirus vectors. in Lentivirus Gene Engineering Protocols (Federico, M., ed), pp. 43–55, Humana Press

Li, G., and Stahl, P. D. (1993) Post-translational processing and membrane association of the two early endosome-associated rab GTP-binding proteins (rab4 and rab5). Arch. Biochem. Biophys. 304, 471–478

Haas, A. K., Fuchs, E., Kopajtich, R., and Barr, F. A. (2005) A GTPase-activating protein controls Rab5 function in endocytic trafficking. Nat. Cell Biol. 7, 887–893

Blümer, J., Rey, J., Dehmelt, L., Mazel, T., Wu, Y. W., Bastiaens, P., Goody, R. S., and Itzen, A. (2013) RabGEFs are a major determinant for specific Rab membrane targeting. J. Cell Biol. 200, 287–300

Sivars, U., Aivazian, D., and Pfeffer, S. R. (2003) Yip3 catalyses the dissociation of endosomal Rab-GDI complexes. Nature 425, 856–859

Ohya, T., Miaczynska, M., Coskun, U., Lommer, B., Runge, A., Drechsel, D., Kalaidzidis, Y., and Zerial, M. (2009) Reconstitution of Rab- and SNARE-dependent membrane fusion by synthetic endosomes. Nature 459, 1091–1097

Kümmel, D., and Ungermann, C. (2014) Principles of membrane tethering and fusion in endosome and lysosome biogenesis. Curr. Opin. Cell Biol. 29C, 61–66

Elias, M., Brighouse, A., Gabernet-Castello, C., Field, M. C., and Dacks, J. B. (2012) Sculpting the endomembrane system in deep time: high resolution phylogenetics of Rab GTPases. J. Cell Sci. 125, 2500–2508

Mishra, A., Eathiraj, S., Corvera, S., and Lambright, D. G. (2010) Structural basis for Rab GTPase recognition and endosome tethering by the C2H2 zinc finger of Early Endosomal Autoantigen 1 (EEA1). Proc. Natl. Acad. Sci. U.S.A. 107, 10866–10871

Wang, L., Liang, Z., and Li, G. (2011) Rab22 controls NGF signaling and neurite outgrowth in PC12 cells. Mol. Biol. Cell 22, 3853–3860

Atkinson, H. A., Daniels, A., and Read, N. D. (2002) Live-cell imaging of endocytosis during conidial germination in the rice blast fungus, Magnaporthe grisea. Fungal Genet. Biol. 37, 233–244