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Expression and localization of exocytic and recycling Rabs from *Magnaporthe oryzae* in mammalian cells

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Abstract

Rab GTPases are master regulators of intracellular membrane trafficking along endocytic and exocytic pathways. In this chapter, we began to characterize the exocytic and recycling Rabs from the filamentous fungus *Magnaporthe oryzae* (*M. oryzae*) that causes the rice blast disease. Among the 11 putative Rabs identified from the *M. oryzae* genome database (MoRabs), MoRab1, MoRab8, and MoRab11 appear orthologs of mammalian Rab1, Rab8, and Rab11 and likely function in exocytosis and endosomal recycling. To test this contention, we cloned, expressed, and determined intracellular localization of the three MoRabs in mammalian cells, in comparison to their human counterparts (hRabs). The MoRabs were well expressed as GFP fusion proteins and colocalized with the tdTomato-labeled hRabs on exocytic and recycling organelles, as determined by immunoblot analysis and confocal fluorescence microscopy. The colocalization supports the contention that the MoRabs are indeed Rab orthologs and may play important roles in the development and pathogenicity of *M. oryzae*.

INTRODUCTION

Rab GTPases are molecular switches that regulate intracellular membrane trafficking on endocytic, exocytic, and recycling pathways (Hutagalung & Novick, 2011; Li & Segev, 2012; Pfeffer, 2013). Each Rab targets to a specific organelle and controls multiple steps of vesicular transport by alternating between GTP-bound “on” conformation and GDP-bound “off” conformation with the assistance of upstream regulators and downstream effectors (Stenmark, 2009). Rab11, Rab8, and Rab1 in particular are involved in endosomal recycling and exocytosis, respectively. Rab11 is localized to so-called slow recycling endosomes mediating transport of internalized receptors via clathrin-dependent endocytosis, e.g., transferrin receptor, back to the plasma membrane (Ullrich, Reinsch, Urbe, Zerial, & Parton, 1996). In polarized cells, Rab11 specifically targets to apical recycling endosomes destined to the apical plasma membrane and plays an important role in the transcytosis of polymeric IgA receptor from basolateral to apical cell surface (Goldenring, Roland, & Lapierre, 2012).

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Rab8 is involved in recycling different populations of cargoes including those internalized via clathrin-independent endocytosis such as MHC I. Interestingly, Rab11 and Rab8 form a Rab-activation cascade in recycling cargoes to specialized cell surface structures such as the primary cilia (Knodler et al., 2010). This recycling Rab cascade is mediated by Rabin8, which is a guanine nucleotide exchange factor (GEF) for Rab8 but also an effector of Rab11 and can be recruited to the recycling endosomes by Rab11 for activation of Rab8 (Knodler et al., 2010). Rab8 is also suggested to deliver newly synthesized membrane cargoes directly to the cell surface via the exocytic pathway, especially in polarized cells (Goldenring et al., 2012). Along the exocytic pathway, there is another Rab, Rab1, which is localized at the endoplasmic reticulum (ER) exit sites and the pre-Golgi intermediate compartment (IC) to mediate ER to Golgi transport (Stenmark, 2009; Taussig, Chen, & Segev, 2012). In addition, Rab1 is also involved in the initiation of autophagy (Lynch-Day et al., 2010; Taussig et al., 2012).

These three recycling and exocytic Rabs are conserved in evolution from the last eukaryotic common ancestor (LECA) to humans (Diekmann & Pereira-Leal, 2013; Elias, Brighthouse, Gabernet-Castello, Field, & Dacks, 2012; Kloppe, Kienle, Fasshauer, & Munro, 2012), suggesting functions fundamental to eukaryotic cells. Here we present methods for cloning and initial localization study of Rab11, Rab8, and Rab1 homologs from *M. oryzae*, a pathogenic filamentous fungus in plants that causes rice blast disease (Ebbole, 2007). Although the three Rabs have multiple isoforms in mammalian cells, they have only one isoform each in *M. oryzae* and are termed here as MoRab11, MoRab8, and MoRab1 among a total of 11 MoRabs identified from the *M. oryzae* genome database. The MoRabs and their human counterparts are coexpressed in mammalian cells as eGFP and tdTomato fusion proteins, respectively, via a bidirectional expression vector, and are found to colocalize to recycling endosomes and exocytic structures. The results suggest that MoRab11, MoRab8, and MoRab1 are authentic orthologs of mammalian counterparts and should help understand the function of endosomal recycling and exocytosis in the development and pathogenicity of *M. oryzae*.

1. MATERIALS AND EQUIPMENT

1.1 REAGENTS FOR CLONING AND PLASMID CONSTRUCTION

Plasmids: pGEM-T Easy vector (Promega), pBI-Tet (Clontech), pTet-Off (Clontech)

Bacterial strains: *E. coli* DH5 α and MC1061

Fungal strain: Guy11

Growth media: For bacteria: LB liquid (Difco) and LB Agar (Difco). For *M. oryzae*: Complete medium plates containing 0.6% yeast extract, 0.6% casein hydrolysate, 1% sucrose, and 1.5% agarose

Ampicillin: 1000x stock solution (100 mg/mL in ddH₂O sterilized by filtration, aliquoted and stored at -20 °C)

Diethylpyrocarbonate (DEPC) (TIANGEN Biotech)

RNAiso and SYBR PrimeScriptTM RT-PCR Kit (Takara)

ImProm-IITM Reverse Transcription System (Promega)

1.2 REAGENTS FOR PROTEIN EXPRESSION AND ANALYSIS

Tissue culture: Baby hamster kidney (BHK) cells (BHK-21 cell line from ATCC)

Growth media: α -minimal essential medium (MEM) (Invitrogen) containing 5% fetal bovine serum (FBS) (Invitrogen), glutamine, penicillin/streptomycin

Lipofectamine 2000 transfection reagent (Invitrogen)

Phosphate-buffered saline (PBS) (Sigma)

1X SDS-loading buffer: 50 mM Tris-HCl (pH6.8), 2% (W/V) SDS, 0.1% (W/V) bromophenol blue, 10% (V/V) glycerol, 1% β -mercaptoethanol (add before use)

Reagents for SDS-PAGE (12% separating gel):

Separating gel		Stacking gel	
40% Acrylamide:Bis	3 mL	40% Acrylamide:Bis	0.5 mL
1.5M Tris-HCl pH8.8	2.5 mL	0.5M Tris-HCl pH 6.8	1.25 mL
10% SDS	100 μ L	10% SDS	50 μ L
Distilled H ₂ O	4.5 mL	Distilled H ₂ O	3.25 mL
Mix well, then add		Mix well, then add	
10% Ammonium persulfate	100 μ L	10% Ammonium persulfate	50 μ L
TEMED	10 μ L	TEMED	5 μ L

Immobilon-P PVDF membrane (Millipore)

Antibodies: anti-GFP monoclonal antibody (mAb) (BD Biosciences), anti-DsRed mAb (Sigma), anti-actin mAb (Sigma), IRDye 800CW goat anti-mouse IgG (LI-COR Biosciences)

1.3 MATERIALS AND REAGENTS FOR CONFOCAL FLUORESCENCE MICROSCOPY

Coverslips: 12 Circular-1 (Fisher Scientific)

Glass Slides: VWR Micro Slides, 25 $\times$ 75 mm 1.0 mm thick

ProLong Gold Antifade Reagent with DAPI (Invitrogen)

16% paraformaldehyde (PFA) (Electron Microscopy Sciences)

1.4 EQUIPMENT

UV spectrophotometer (GeneQuant, GE Healthcare)

Centrifuge (5415R, Eppendorf)

CO₂ tissue culture incubator (Fisher Scientific)

Platform shaker (New Brunswick Scientific)

Bio-Rad Semi-Dry Transfer Apparatus

Odyssey Infrared Imaging System (LI-COR Biosciences)

Leica SP2 MP confocal laser scanning microscope

2. METHODS

2.1 CLONING OF MoRab11, MoRab8, AND MoRab1

2.1.1 Extraction of total RNA from the *M. oryzae* strain Guy11

1. Guy11 strain was grown in 100 mL liquid complete medium (CM) for 3 days, and the mycelium body was harvested with a filter paper, frozen in liquid nitrogen, and grounded into powder.
2. The powder was transferred to a 1.5-mL Eppendorf centrifuge tube (DEPC-treated) to which 1 mL RNAiso reagent was added and mixed by vortexing for 30 sec, followed by incubation at room temperature for 5 min.
3. Add 200 μ L of chloroform to the tube and mix well by vortexing.
4. Centrifuge at 12,000 rpm for 15 min at 4 °C.
5. Transfer the supernatant to a new 1.5 mL tube (DEPC-treated), and add 0.6 volume of isopropanol. Mix well and incubate at room temperature for 20 min.
6. Centrifuge at 12,000 rpm for 15 min at 4 °C.
7. Aspirate the supernatant, wash the pellet twice with 70% ethanol, and air-dry at room temperature for 20 min.
8. Add 200 μ L RNase-free H₂O to dissolve the pellet, then add 2 μ L 5 mg/mL DNase I and incubate at 37 °C for 1 h.
9. Inactivate the DNase I by incubation at 65 °C for 20 min.
10. Add RNase-free H₂O to bring the total volume up to 400 μ L, then add an equal volume of H₂O-saturated phenol: chloroform: isoamyl alcohol mixture (25:24:1).
11. Centrifuge at 12,000 rpm for 15 min at 4 °C.
12. Transfer the supernatant to a new 1.5 mL tube (DEPC-treated), add 2 volume of ethanol and 0.1 volume of 3M NaAc, mix well and incubate at –80 °C for 2 h.
13. Centrifuge at 12,000 rpm for 15 min at 4 °C.
14. Aspirate the supernatant, wash the pellet twice with 70% ethanol, and air-dry at room temperature for 20 min.
15. Add 50 μ L RNase-free H₂O to dissolve the pellet and store the RNA preparation at –80 °C.
16. Measure the RNA concentration and purity by using a GeneQuant spectrophotometer.

2.1.2 Amplifying the cDNAs of MoRab11, MoRab8, and MoRab1 by RT-PCR and cloning into the bidirectional expression vector pBI-Tet with human Rab counterparts

1. The *M. oryzae* RNA extract was used as template for the synthesis of cDNAs of MoRab11 (MGG_01079), MoRab8 (MGG_06135), and MoRab1 (MGG_06962) by RT-PCR using the SYBR PrimeScript™ RT-PCR Kit. The resulting cDNAs were cloned into the pGEM-T Easy vector and confirmed by direct DNA sequencing.
2. The cDNAs of MoRab11, MoRab8, and MoRab1 were amplified by PCR and cloned into the *NotI/SaI* sites of the pBI-Tet expression vector, in-frame with the upstream eGFP cDNA previously cloned at the *PstI/NotI* sites. The three resulting plasmid constructs are named: pBI/eGFP-MoRab11, pBI/eGFP-MoRab8, and pBI/eGFP-MoRab1.
3. The pBI-Tet vector is a bidirectional expression vector and can express a second gene cloned at the *MluI/NheI* sites. To this end, the cDNAs of human Rab counterparts and Rab5 as control were cloned into the *MluI/NheI* sites and the tdTomato (tdTom) cDNA was cloned in-frame upstream of the *MluI* site. As such, eight expression constructs were generated, which are as follows (Figure 1):

pBI/eGFP-MoRab11/tdTom-hRab11

pBI/eGFP-MoRab11/tdTom-hRab5

pBI/eGFP-MoRab8/tdTom-hRab8

pBI/eGFP-MoRab8/tdTom-hRab5

pBI/eGFP-MoRab1/tdTom-hRab1

pBI/eGFP-MoRab1/tdTom-hRab5

2.2 EXPRESSION AND INTRACELLULAR LOCALIZATION OF MoRab11, MoRab8, AND MoRab1

2.2.1 Transfection of BHK-21 cells

1. BHK-21 cell monolayers were grown in either 6-well culture plates or 24-well culture plates with coverslips in α -MEM containing 5% heat-inactivated FBS and 20 U/mL penicillin/streptomycin, for immunoblot analysis of protein expression and confocal fluorescence microscopy, respectively. The cells were seeded and then incubated for 24 h in a 37 °C tissue culture incubator with 5% CO₂ for the cell monolayer to reach ~70–80% confluence before transfection.
2. Each aforementioned expression construct (2 μ g or 0.4 μ g for 6-well and 24-well plates, respectively) was cotransfected with pTet-Off (2 μ g or 0.4 μ g) into each well of cells using the Lipofectamine 2000-mediated transfection procedure. In the experiments for immunoblot analysis or confocal microscopy, the plasmid DNAs were mixed in 250 μ L or 50 μ L of α -MEM in a 1.5 mL Eppendorf tube,

and 10 μ L or 2 μ L of Lipofectamine 2000 were mixed in 250 μ L or 50 μ L of α -MEM in a separate tube. Incubate at room temperature for 4 min. Then transfer the DNA mix to the Lipofectamine 2000 mix, and incubate at room temperature for 20 min. Then add the 500 μ L or 100 μ L of DNA-Lipofectamine 2000 complexes dropwise to the cells in 6-well or 24-well plates.

3. Place the cells back in the 37 °C incubator for 5 h.
4. Replace the transfection medium with full growth medium, and continue the incubation for another 24 h.

2.2.2 Immunoblot analysis of protein expression

1. Aspirate the medium and wash the cell monolayer once with 1 mL of PBS.
2. Add 200 μ L of 1X SDS-loading buffer directly to each well, grind the cells off the plate with a syringe plug.
3. Transfer the cell lysate to a 1.5 mL tube with a syringe, and pass through a 25G3/8 needle 10 times to shear the nuclear DNA and reduce stickiness.
4. Boil the cell lysate for 3 min, then run 20 μ L on SDS-PAGE (12% gel).
5. Transfer the proteins from the gel to a PVDF membrane by using the Bio-Rad Semi-Dry Transfer apparatus.
6. Probe the membrane with the primary antibodies for GFP (1:1000 dilution), DsRed (1:1000 dilution), and actin (1:500 dilution), respectively, followed by the secondary antibody (IRDye 800CW goat anti-mouse IgG) at 1:15,000 dilution.
7. Visualize and quantify the labeled proteins on the membrane by using an LI-COR Odyssey Infrared Imaging System, with manufacturer's analytical software (Figure 1).

2.2.3 Localization by confocal fluorescence microscopy

1. Aspirate the medium and wash the cell monolayer on coverslip once with 0.5 mL of PBS.
2. Add 0.5 mL of freshly made 4% PFA to fix the cells in the 37 °C CO₂ incubator for 15 min.
3. Aspirate the fixation solution, and wash the cell monolayer on coverslip once with 0.5 mL PBS.
4. Pick up the coverslip with a pair of fine forceps, and mount it onto 10 μ L of ProLong Gold Antifade Reagent with DAPI on a glass slide. Make sure that the cell monolayer faces the mounting reagent on the slide.
5. Keep the slide in the dark and at room temperature overnight to dry the residual liquid.
6. Observe and analyze the intracellular localization of fluorescent eGFP-MoRabs and tdTomato-hRabs in the cell by a Leica confocal laser scanning microscope

with Ar-488 and Kr-568 laser excitation and associated analytical software (Figure 2).

SUMMARY

In this chapter, we have described the experimental protocols for cloning and subcellular localization of recycling and exocytic Rabs from the rice blast fungus *M. oryzae*, including MoRab11, MoRab8, and MoRab1. Each MoRab is coexpressed with a human counterpart (hRab) in mammalian cells as eGFP- and tdTomato-tagged fluorescent proteins, respectively. The bidirectional expression vector pBI-Tet is used to express a MoRab and a hRab simultaneously in the same cell to facilitate the single-cell assay and confocal fluorescence microscopy analysis of their colocalization. Our results show that MoRab11, MoRab8, and MoRab1 colocalizes with hRab11, hRab8, and hRab1, respectively, on recycling endosomes and exocytic organelles, but not with hRab5 that is associated with early endosomes to promote endocytic traffic to late endosomes and lysosomes. The results suggest that MoRab11, MoRab8, and MoRab1 are indeed orthologs of mammalian Rab11, Rab8, and Rab1 and may play the same roles in recycling endocytosed receptors and cargoes and transporting newly synthesized membrane proteins to the plasma membrane in *M. oryzae*. The experimental protocols described here may be extended for cloning and validation of additional MoRabs to understand the function of intracellular membrane trafficking in the development and pathogenicity of *M. oryzae*.

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FIGURE 1. Schematic structure of the pBI-Tet bidirectional expression vector and coexpression of eGFP-labeled MoRabs with tdTomato-labeled hRabs in BHK cells
 (A) Schematic diagram of the pBI-Tet constructs with eGFP-MoRabs (Green and light gray) cloned at the *NotI/SalI* sites in the MCSII region and tdTomato-hRabs (Red and dark gray) cloned at the *MluI/NheI* sites in the MCSI region. Upon cotransfection with pTet-Off, the pBI-Tet construct may coexpress an eGFP-MoRab and a tdTomato-hRab, via the TRE region and promoter. (B) Immunoblots of eGFP-MoRabs (G-MoRabs) and tdTomato-hRabs (T-hRabs). The cell lysates were subjected to SDS-PAGE and immunoblot analysis with the antibody for eGFP, antibody for DsRed that recognizes tdTomato, and antibody for actin that serves as an internal loading control. Molecular mass standards (in kDa) are indicated on the left side of the panels.

FIGURE 2. Colocalization of MoRab1, MoRab8, and MoRab11 with hRab1A/B, hRab8, and hRab11 in BHK cells

(A) Shown are confocal fluorescence images of intracellular structures in BHK cells labeled by eGFP-MoRabs (green) and tdTomato-hRabs (red), as indicated. The early endosomal hRab5 here serves as a negative control versus recycling endosomes. Nuclei are identified with DAPI staining (blue). The results are reproducible in three independent experiments. Bar = 10 μ m. (B) Quantification of colocalization between the eGFP-MoRabs and the coexpressed tdTomato-hRabs in the same cells. The graph shows Pearson's correlation coefficient between each eGFP-MoRab and the corresponding tdTomato-hRabs, which is calculated using the confocal fluorescent images from four cells and the colocalization tool in the Velocity software. Shown are the mean and calculated SEM. (See color plate)