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Role of Rab1b in COPII dynamics and function

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ABSTRACT

In eukaryotic cells, proteins destined for secretion are translocated into the endoplasmic reticulum (ER) and packaged into so-called COPII-coated vesicles. In the ER exit sites (ERES), COPII has the capacity of deforming the lipid bilayer, where it modulates the selective sorting and concentration of cargo proteins. In this study, we analyze the involvement of Rab1b in COPII dynamics and function by expressing either the Rab1b negative-mutant (Rab1N121I) or the Rab1b GTP restricted mutant (Rab1Q67L), or performing short interference RNA-based knockdown. We show that Rab1b interacts with the COPII components Sec23, Sec24 and Sec31 and that Rab1b inhibition changes the COPII phenotype. FRAP assays reveal that Rab1b modulates COPII association/dissociation kinetics at the ERES interface. Furthermore, Rab1b inhibition delays cargo sorting at the ER exit sites. We postulate that Rab1b is a key regulatory component of COPII dynamics and function.

Introduction

Intracellular transport between the compartments of the secretory pathway relies on a series of protein-sorting events performed by coat protein complexes (COPs), among others. These complexes carry out essential roles, such as the selecting and recruiting of cargo proteins, as well as deforming the lipid bilayer of the donor membrane into buds and vesicles (Lee and Miller, 2007; Sato and Nakano, 2007). Coat protein complex II (COPII) is essential for protein export from ER exit sites (ERES) (Bannykh et al., 1996; Orci et al., 1991), and is composed of the small GTPase Sar1 and two hetero-dimeric large protein complexes Sec23/Sec24 and Sec13/Sec31 (Barlowe et al., 1994; Matsuoka et al., 1998). COPII vesicle formation involves several steps. First, Sec12, an ER-localized transmembrane protein, catalyzes GDP/GTP exchange on Sar1 (Barlowe et al., 1993). Then, GTP loaded Sar1 recruits the cargo adaptor complex Sec23/Sec24 to ER exit domains. Sec23 is the GTPase activating protein (GAP) that stimulates the enzymatic activity of Sar1 (Yoshihisa et al., 1993), whereas Sec24 is the adaptor protein that captures specific transmembrane cargos and SNAREs in the nascent vesicle (Kuehn et al., 1998; Miller et al., 2002; Mossessova et al., 2003). Finally, the "pre-budding" complex Sar1-GTP/Sec23p/Sec24p recruits the Sec13p/Sec31p complex, which provides the outer layer of the coat (Lederkremer et al., 2001; Stagg et al., 2006) and supports further stimulation of GTP hydrolysis and the consequent release of Sar1 (Antonny et al., 2001). Sequential interactions between the different COPII subunits give rise to coat polymerization, selective cargo recognition and sorting, membrane curvature, and the release of small transport carriers from the ER membrane.

After budding from the ER, the COPII vesicles rapidly fuse homotypically (Xu and Hay, 2004) to subsequently generate COPI-coated membrane structures called vesicular–tubular clusters (VTC-(Bannykh et al., 1996)). These pleiomorphic transport carriers containing secretory cargo move towards the Golgi complex along the microtubule-based cytoskeleton and fuse with the cis-cisterna of the Golgi apparatus (Bannykh et al., 1996).

COPII coat not only selects membrane cargo, but also has the ability to recruit the TRAPPI complex through the interaction between the Sec23p COPII subunit and Bet3 (Cai et al., 2007; Sacher et al., 2001). In yeast, TRAPPI functions as a guanine nucleotide exchange factor (GEF) for the small GTPase Ypt1p (Sacher et al., 2001; Wang et al., 2000). By recruiting TRAPPI, and thus activating Ypt1p, the COPII vesicle coat has the ability to integrate the vesicle formation process with its tethering and fusion with Golgi membranes (Cai et al., 2007). Consequently, the sequential activation of downstream fusion machinery ensures the specificity of vesicle delivery with the target organelle (Cai et al., 2007; Kim et al., 2006).

Rab1a and Rab1b isoforms are Ypt1p mammalian homologues (Touchot et al., 1989), localized at the endoplasmic reticulum–Golgi interface and also within the Golgi complex (Plutner et al., 1991; Saraste et al., 1995) and are required for the transport of membranes from the ER to the Golgi (Tisdale et al., 1992). Rab1 binds to several effectors, and its role in transport has been largely attributed to the tethering and fusion steps (Allan et al., 2000; Moyer et al., 2001; Satoh et al., 2003; Weide et al., 2001). Furthermore, Rab1b recruits GBF1 (Monetta et al., 2007), a GEF for

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Arf1 (Claude et al., 1999; Kawamoto et al., 2002), which modulates COPI membrane association at the ER-Golgi interface (Garcia-Mata et al., 2003). In the present work, we describe the contribution of Rab1b activity to COPII dynamics and function. We show that Rab1b interacted with the COPII components Sec23, Sec24 and Sec31. Inhibition of Rab1b expression caused a change in the COPII phenotype and a delay in the transport of ER resident enzymes at the ERES interface level. Furthermore, FRAP assays revealed that the Rab1b active mutant, Rab1Q67L, stabilized COPII associated to punctuated structures, suggesting that Rab1b could be a key regulatory component in COPII turnover and stability in the membrane.

Materials and methods

DNA constructs and antibodies

GFP-Rab1b, Rab1b-myc constructs, and Rab1b mutants (Rab1N121I and Rab1Q67L) were previously described in Alvarez et al. (2003). GST-Rab1b versions were subcloned from their respective Rab1b-myc constructs into pGEX4T-2- (Amersham, Biosciences, Piscataway, NJ). Sec13-YFP was kindly supplied by Dr. Benjamin Glick (University of Chicago, IL), GFP-Sec16 by Dr. David Stephens (University of Bristol, UK) and GalT2-YFP and ManII-CFP by Dr. Hugo Maccioni (National University of Córdoba, Argentina). The GST-Rab5 vector was gratefully provided by Dr. Marisa Colombo (National University of Cuyo, Argentina). Full-length Sec24C was obtained by PCR, using as template the RZPD clone ID IRAUp969A0555D6 (RZPD German Resource Center for Genome Research). The primers used to amplify full-length Sec24C were 5′-TGCTGTCGACATGAACGTCAACCAGTCAG-3′ (forward primer) and 5′-CCACCGCGGGCTCAGTAGCTGCCGAATC-3′ (reverse primer). Full-length Sec24C (Gen Bank accession number BC018928) was cloned into the SalI–SacII restriction sites of the pEYFP-C1 vector (BD Biosciences Clontech, San Jose, CA).

The following antibodies were used: rabbit polyclonal antibody to p115 (Nelson et al., 1998) (a gift from Dr. Elizabeth Sztul, University of Alabama at Birmingham, Al). Mouse monoclonal antibody and rabbit polyclonal antibody to GFP and mouse monoclonal antibody to GM130 and to Sec24D (Abcam, Cambridge, MA); rabbit polyclonal antibody to Calreticulin (Affinity BioReagents, Golden, CO); goat polyclonal antibody to Sec23 and rabbit polyclonal antibody to Rab1b and Rab1a (Santa Cruz Biotechnology, Santa Cruz, CA); mouse monoclonal antibody to Sec31A (BD Biosciences, San Jose, CA) and to Sec24D (Abnova, Walnut, CA); goat anti-mouse Alexa Fluor 594 and goat anti-rabbit Alexa Fluor 488 (Invitrogen, Carlsbad, CA).

Cell culture, DNA transfection, BFA treatment and immunofluorescence assays

HEK293T cells were grown in DMEM with high glucose supplemented with 10% FBS, 100 μg/ml penicillin and 100 μg/ml streptomycin, and plated onto either sterile microscopy glass coverslips (Fisher Scientific, Pittsburgh, PA) or WillCo glass bottom dishes (Warner Instruments, Hamden, CT). At about 70% confluence, cells were transfected using Transit LTI (Mirus, Madison, WI) according to the manufacturer's instructions. After 48 h, cells were fixed for immunofluorescence or processed for *in vivo* studies. Fixation and staining of cells were performed as described (Alvarez et al., 2003). Immunofluorescences were analyzed using a Zeiss LSM Pascal (Carl Zeiss, Thornwood, NY) or FV300 Olympus (Olympus Latin America, Miami, FL) confocal microscopes. For CFP analysis in GFP-expressing cells, excitation was achieved using the 458-nm line of an argon laser and CFP emission was acquired between 480 and 495 nm. Under these conditions, GFP emission was not detected. GFP analysis in CFP expressing cells is described below in FRAP assay section.

In BFA assays, HEK293T cells were plated on glass coverslips for 24 h before treatment with BFA (2.5 μg/ml) for 45 min at 37 °C.

COPII structure quantification was performed using ImageJ processing software (www.rsb.info.nih.gov/ij). Briefly, RGB images were converted to an 8-bit grayscale and a threshold was applied to eliminate the background. Structure quantification and size distribution were accomplished using the Analyze particles and distribution functions.

Expression and purification of GST fusion proteins

BL21 cells were transformed with plasmids encoding different proteins fused to GST. The GST protein expression and binding to glutathione-sepharose 4B (GS4B) beads were performed according to the manufacturer's instructions (Amersham Biosciences, Piscataway, NJ). Beads containing immobilized GST-Rab1b or GST-Rab5 were processed as described in (Christoforidis and Zerial, 2000).

Rat liver cytosol preparation and pull down assays

Rat liver cytosol (RLC) was prepared as described previously (Monetta et al., 2007) and precleared with GS4B beads. For pull down assays, the GST-Rabs:GTPγS or GST-Rabs:GDP attached to the beads was incubated with RLC for 2 h at 4 °C in the presence of 100 μM GTPγS or 100 μM GDP. Beads were then washed four times with NS buffer containing 0.1% Triton X-100, 1 mM DTT, 100 μM GTPγS or GDP. Interacting proteins were analyzed by Western blot, using anti-GFP, anti-p115 and anti-Sec23 antibodies.

In vivo co-immunoprecipitation assay

HEK293T cells were grown in DMEM high glucose supplemented with 10% FBS, 100 μg/ml penicillin and 100 μg/ml streptomycin, and transfection of GFP-Rab1b or GFP-Rab7 was performed using Transit LTI (Mirus, Madison, WI). After 48 h, cells were washed twice with PBS, lysed in 150 μl of RIPA buffer (150 mM NaCl, 1% NP 40, 0.5% Deoxicolato de Na, 0.1% SDS, 50 mM Tris–HCl pH 8) supplemented with Complete-EDTA free protease inhibitors (Roche, Mannheim, Germany), and incubated for 30 min on ice prior to centrifugation at 12,000 × *g* for 10 min at 4C. The supernatant fraction was incubated with 40 μl of PBS washed G protein to perform the preclear step. Finally, 450 μl of PBS was added to the supernatant, which was then incubated in PBS buffer containing 1 mM GTPγS for 30 min at room temperature.

Mouse monoclonal antibody to GFP (20 ng) was incubated with 40 μl of PBS washed G protein beads for 1 h at room temperature, then washed with PBS and crosslinked using Dimethyl Pimelimidate 2HCl (DMP, Pierce, Rockford, IL) for 30 min at room temperature. The reaction was stopped by incubating the antibody-G protein complex (ab-G) in 300 μl of Tris–HCl 100 mM (pH 8.0). The ab-G protein complex was incubated with the supernatant for 2 h at 4 °C and washed 4 times with PBS containing 100 μM GTPγS. Interacting proteins were separated by 10% SDS-PAGE and analyzed by Western blot, using antibodies against Sec23, p115, Sec24 and Sec31 antibodies.

FRAP assays

For Sec13-YFP FRAP analysis, a prebleach image was taken at 0.05% laser intensity. Then, the region of interest (ROI) was bleached for 3 s at 100% laser intensity, with fluorescence recovery in the bleached area being monitored by scanning at 0.05% laser intensity every 10 s or 20 s interval. At the same time periods, the intensity of a reference region (RER) was considered. The RER was an