SUPPLEMENTARY MATERIALS AND METHODS

Plasmids

Wild-type and point mutant (G172E) BARS tagged with a 6x-his epitope were generated in the expression vector pET-11a, while other BARS mutants tagged with the 6x-his epitope were generated in pET-15b (both vectors from Novagen, Madison, WI). Wild-type and CTP mutant BARS were also subcloned into the mammalian expression vector pcDNA3 by inserting into XbaI and NotI sites. GST fused to ARFGAP1 or BARS was constructed in the pGEX-4T-3 expression vector (Amersham Biosciences, Piscataway, NJ). Proteins were purified according manufacturer's instructions, with 6x-his-tagged BARS using nickel beads (Qiagen, Valencia, CA) and GST-ARFGAP1 and GST-BARS using glutathione-coated Sepharose beads (Amersham Biosciences, Piscataway, NJ). VSVG(ts045) and VSVG(ts045)-KDELR have been described previously (Cole et al., 1998) (from J. Lippincott-Schwartz, NIH, Bethesda, MD).

Antibodies

Primary antibodies used included: mouse anti-β-COP antibody (M3A5), rabbit anti-ε-COP and anti-ζ-COP antibodies (from J. Rothman and T. Sollner, Memorial Sloan Kettering Cancer Center, New York, NY), rabbit anti-ARFGAP1 antibody (from D. Cassel, Technion Institute of Technology, Haifa, Israel), rabbit anti-fibronectin antibody (Sigma, St. Louis, MO), mouse anti-membrin antibody (from W. Hong, Institute of Molecular and Cellular Biology, Singapore), mouse anti-VSVG antibody (BW8G65), mouse anti-myc antibody (9E10), rabbit anti-6xHis antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Sheep antibodies against p115 and GRASP55, and rabbit antibodies against GRASP65 and mannosidase I were kindly

provided by F. Barr (Max-Planck Institute of Biochemistry,Martinsried, Germany). The rabbit anti-BARS antibody, p50-2, was raised against GST-BARS and purified as described previously (Spano et al., 1999).

Secondary antibodies used included: Cy2 or Cy3 conjugated donkey antibodies against either mouse IgG or rabbit IgG (Jackson ImmunoResearch, West Grove, PA), horseradish peroxidase conjugated donkey antibodies against mouse IgG, rabbit IgG, rat IgG, or sheep IgG (Jackson ImmunoResearch, West Grove, PA), 10 nm gold-conjugated goat antibodies against either mouse IgG or rabbit IgG (Sigma, St. Louis, MO).

Vesicle reconstitution system

The first-stage incubation contained Golgi membrane (0.2 mg/ml) prewashed with salt condition as indicated, coatomer (6 ug/ml), ARF1 (6 ug/ml), and 2 mM GTP in 500ul of assay buffer (25mM Hepes-KOH, pH7.2, 50mM KCl, 2.5mM $Mg(OAc)_2$, 1mg/ml soybean trypsin inhibitor, 1 mg/ml BSA, and 200mM sucrose) for 15 minutes at 37˚C. Reactions were stopped by incubating in an ice water bath for 5 min, and samples were centrifuged for 10 minutes at 12,000 x g and 4˚C to re-pellet the Golgi membrane. This pellet was then resuspended in 100 ul of assay buffer for the second-stage incubation, which contained ARF-GAP1 (6 ug/ml) and BARS (10 ug/ml), for 10 minutes at 37˚C. Reactions were again stopped by incubation in an ice water bath for 5 minutes, followed by centrifugation for 10 minutes at 12,000 x g at 4˚C to collect the pellet fraction that contained Golgi membrane and the supernatant fraction that contained COPI vesicles. Mutant BARS were used at same molar level as wild-type form. All BARS used for the reconstitution system contained the 6xHis tag at the carboxy terminus.

For velocity sedimentation of the supernatant from the second-stage incubation, ultracentrifugation was performed at 200,000 x g for 1 hour using a SW55 rotor (Beckman Coulter, Fullerton, CA). To analyze COPI vesicles by equilibrium centrifugation, the reactions were scaled up five-fold, and ultracentrifugation was performed at 200,000 x g for 16 hours, as described previously (Yang et al., 2002). Quantitation was performed by first scanning films of immunoblots with a Perfection 1200U flatbed scanner (Epson, Long Beach, CA) followed by analysis using imaging software (Scion Corporation, Frederick, MD).

Electron microscopy

EM examination of Golgi membrane after different stringencies of washing was performed by first embedding in epon followed by serial sections for visualization. EM examination of reconstituted vesicles or Golgi membrane after the two-stage incubation system by whole-mount on Formvar grids, either with or without immunogold labeling, was performed as described previously (Yang et al., 2002). Quantitation was performed as described in individual figure legends.

Knockdown by siRNA

The siRNA sequences are: 5'-GGAUAGAGACCACGCCAGUUU-3' against BARS (from Dharmacon, Lafayette, CO) and 5'-AGAUGAACUUACCGUAGAA-3' against Xrn1 (from Ambion, Austin, TX). The siRNA constructs were expressed in COS-7 cells using Oligofectamine (Invitrogen, Carlsbad, CA).

In vivo transport of VSVG constructs

To assess the effect of different forms of BARS, COS-7 cells were transiently transfected by FuGene6 (Roche, Indianapolis, IN) with VSVG(ts045)-KDELR and either with or without different forms of BARS at 37°C for 24 hours, and then incubate at 32°C for 16 hours for the permissive condition. Cells were then shifted to 40°C for 1 hour for the non-permissive condition.

To assess the effect of different siRNAs, COS-7 cells were incubated with different siRNAs and Oligofectamine at 37°C for 24 hours, and then either VSVG(ts045)-KDLER or VSVG(ts045) were transfected for another 24 hours. To measure retrograde transport, cells that expressed VSVG(ts045)-KDELR were subjected to temperature shifts as mentioned above.

To measure anterograde transport, cells that expressed VSVG(ts045) were shifted to 40^{o}C for 2 hours to accumulate a pool at the ER, and then shifted to 20^{o}C for 20 minutes.

Pull down assays

All incubations were performed at room temperature for 1 hour in 25 mM Hepes-KOH, pH 7.2, 50 mM KCl, 2.5 mM $Mg[OAc]_2$, and 1 mg/ml BSA. To detect an interaction between BARS and GAP, GST-GAP (1 ug) on glutathione-Sepharose beads (Amersham Biosciences, Piscataway, NJ) was incubated with soluble BARS (1 uM). To detect an interaction between BARS on beads and other soluble proteins, GST-BARS (1 ug) was incubated with 6x-his tagged forms of soluble proteins (1 uM). In experiments that involved either palmitoyl-CoA (10uM) or NADH (10uM), these cofactors were pre-incubated with soluble BARS at 37°C for 30 minutes, before being incubated with GST fusion proteins on beads at room temperature for 1 hour. After the incubations, beads were washed six times with the incubation buffer and then analyzed by

SDS-PAGE followed by Western blotting using chemiluminescence (NEN Life Sciences, Boston, MA).

References

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