SUPPLEMENTARY INFORMATION

INVOLVEMENT OF THE Rho/Rac FAMILY MEMBER RhoG IN CAVEOLAR ENDOCYTOSIS

by

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SUPPLEMENTARY MATERIALS AND METHODS

Expression vectors. pCEFL-AU5 vectors encoding RhoG (pAM3), $RhoG^{Q61L}$ (pCEFL-AU5-$RhoG^{Q61L}$), Rac1 (pCEFL-AU5- Rac1), $Rac1^{Q61L}$ (pCEFL-AU5- $Rac1^{Q61L}$) and the $RhoG^{Q61L}$/Rac1 chimera G (pRMP27) have been previously described (Prieto-Sanchez & Bustelo, 2003; Schuebel et al., 1998). pCEFL-AU5 vectors encoding the F37L+Q61L and Y40C+Q61L mutants of both Rac1 and RhoG were generated using the Quickchange mutagenesis kit (Stratagene). pcDNA3-HA-dynamin2 and pcDNA3-HA-$dynamin2^{K44A}$ were provided by Dr. M. A. del Pozo (CNIC, Madrid, Spain). GFP-tagged versions of Rab5 (pHML42), Rab7 (pHML39) and Rab11 (pHML40) were generated by PCR using as template a human leukemia cDNA library. pEGFP vectors were purchased from BD Biosciences Clontech. Mutagenesis to generate constitutively active or dominant negative Rab mutants was performed using the Quickchange kit. The expression vector encoding the caveolin1-EGFP chimeric molecule was a gift from Dr. B. van Deurs (University of Copenhagen Panum Institute, Denmark). The expression vectors encoding HA-Arf6, HA-$Arf6^{Q67L}$, or HA-$Arf6^{T27N}$ were provided by Dr. C. D'Souza-Schorey (University of Notre Dame, IN, USA). The pGEX vector containing the RhoG binding domain of ELMO (amino acid residues 1-362) (Katoh & Negishi, 2003) was obtained from Dr. M. Negishi (Kyoto University, Japan).

Tissue culture conditions. Cells were grown under standard temperature/CO_2 conditions in DMEM supplemented with 1% L-glutamine, 1% penicillin/streptomycin, and 10% calf serum. All tissue culture reagents were obtained from Invitrogen (Carlsbad, CA, USA).

Antibodies and reagents. GTPases were detected by either immunofluorescence or immunoblotting techniques using an anti-AU5 monoclonal antibody (Covance) (1:1,000 dilution). Anti-caveolin and anti-GM130 antibodies were from BD Biosciences and used at 1:200 and 1:100 dilutions, respectively. Anti-clathrin antibodies were from Santa Cruz Biotechnology (1:100 dilution). Anti-dynamin2 antibodies were purchased from Abcam (1:1000 dilution). Anti-calreticulin antibodies were from Calbiochem-Novabiochem (1:200 dilution). Anti-RhoG antibodies were kindly provided by Dr. P. Fort (Centre de Recherche en Biochimie Macromoleculaire, CNRS-UPR1086, 34293 Montpellier Cedex 5, France, EU) and used at a 1:50 dilution. Fluorochrome-labeled secondary antibodies to mouse and rabbit IgGs were from Jackson Immunoresearch Europe. Mytotracker, Lysotracker, Nile Red, and rhodamine-labeled phalloidin were from Molecular Probes. All these reagents were used according to manufacturer's instructions. Horseradish peroxidase-conjugated secondary antibodies were from Amersham Biosciences AG.

Immunofluorescence. Cells were grown on coverslips introduced onto 6-well plates (50,000 cells/well). Transfections were performed with liposomes (FuGENE-6; Roche Molecular Biochemicals, Mannheim, Germany). We used either 1 μg of vectors (for single transfections) or 0.5 μg (for double transfections) of each expression plasmid. 30 hr after transfection, cells were fixed with 3.7% formaldehyde in PBS for 15 min. However, when CTxB was used, cells were incubated with this bacterial toxin for the indicated periods of time and fixed thereafter as indicated above. When appropriate, cells were incubated before CTxB addition with methyl-β-cyclodextrin (20 mM; Sigma-

Aldrich) for 30 min at 37 ºC. In both cases, cells were permeabilized with PBS containing 0.5% Triton X100 for 15 min and blocked in a 25 mM Tris-buffered solution containing 2% bovine serum albumin (Sigma-Aldrich), 0.1% sodium azide (Sigma-Aldrich) and 0.1% Triton X100 for 10 min. Cells were incubated with the appropriate combination of primary and secondary antibodies, mounted onto microscope slides using Mowiol (Calbiochem-Novabiochem), and subjected to immunofluorescence analysis. Antibody dilutions for immunofluorescence were made in blocking solution. Secondary antibodies were used at 1:150 (for Cy2-labeled antibodies), 1:800 (for Cy3-labeled antibodies) or 1:400 (for Cy5-labeled antibodies) dilutions. Fluorescence images were captured with a confocal microscope (Zeiss LSM510) using an ^{18}Ar laser (excitation wavelength: 488 nm) for Cy2, a ^{2}He/^{10}Ne laser (excitation wavelength: 543 nm) for Cy3 and a ^{2}He/^{10}Ne laser (excitation-wavelength: 633 nm) for Cy5. Confocal image analysis was carried out with the LSM 5 Image Browser program (version 2.8, Zeiss, Oberkochen, Germany). Final editing and composition of figures was done using the Canvas 8.0.5 software (ACD Systems, Victoria, BC, Canada).

SUPPLEMENTARY FIGURE LEGENDS

SUPPLEMMENTARY FIGURE 1. Studies on the possible localization of AU5-RhoG^{Q61L} with markers of the clathrin pathway and macropinocytosis. COS-1 cells were transfected with a plasmid encoding AU5-RhoG^{Q61L} either alone (upper panels) or in combination with expression vectors encoding EGFP-Rab and HA-tagged Arf6 proteins. In the case of EGFR panel (top panel on the right), the transfected cells were incubated with EGF at 4 ºC for 30 min and then shifted to 37 ºC for 5 min to allow the internalization of the EGF/EGFR complex. After that step, cells were fixed and incubated with anti-AU5 (green) and EGFR antibodies (red). In the rest of panels, the transfected cells were fixed, stained with anti-AU5, clathrin, or HA antibodies and subjected to confocal microscopy analysis. RhoG^{Q61L} is shown in either green (upper panels) or red (rest of panels) color. The other proteins are visualized with the opposite fluorochrome. Areas of colocalization are shown in yellow.

SUPPLEMENTARY FIGURE 2. RhoG is localized in lipid rafts of the plasma membrane. COS-1 cells expressing AU5-RhoG were subjected to FITC-CTxB internalization experiments as indicated in Materials and Methods. After 5 min of incubation with the toxin at 37 ºC, cells were stained with anti-AU5 and subjected to confocal microscope analysis. The localization of RhoG (middle panel) and FITC-CTxB (left panel) is shown in red and green, respectively. The areas of colocalization of FITC-CTxB with RhoG are shown in yellow (right panel). Arrows indicate areas with membrane ruffles containing both CTxB and RhoG.

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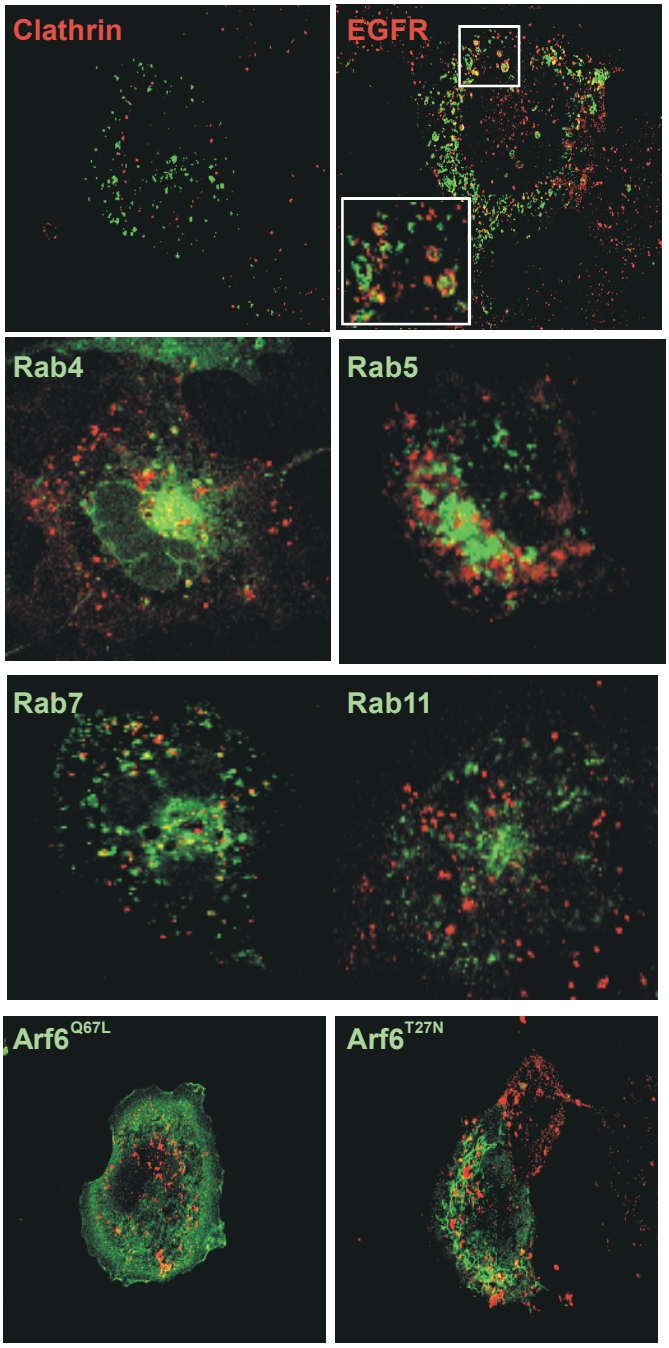

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