Sorting Nexin-1 Mediates Tubular Endosome-to-TGN Transport through Coincidence Sensing of High-Curvature Membranes and 3-Phosphoinositides

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Supplemental Experimental Procedures

Electron Microscopy

The colocalization of SNX1 and CI-MPR was studied via double immunogold labeling of ultrathin cryosections. These studies were performed in HepG2 cells because these show high endogenous levels of both proteins. Cells were fixed with 4% freshly prepared formaldehyde in 0.1 M phosphate buffer (pH 7.4), added to an equal volume of culture medium for 10 min, followed by post-fixation in 4% formaldehyde without medium, and stored at 4°C. Processing of cells for ultrathin cryosectioning and double immunolabeling according to the protein A-gold method was done as described [S1, S2].

Liposome Tubulation and Sedimentation

Liposomes consisting of total bovine brain lipids (Folch fraction 1, Sigma B1502) were resuspended by sonication at 1 mg/ml in 20 mM HEPES, (pH 7.4), 150 mM NaCl, 1 mM DTT and sized by extrusion [S3]. For sedimentation assays, 5 μM protein was incubated with 0.6 g/l liposomes for 5 min before sedimentation at 140,000 $\times$ g in a Beckman TLA100 rotor. Supernatants were immediately removed and pellets were resuspended in an equal volume of buffer before SDS-PAGE analysis. The proteins were visualized by Coomassie stain, imaged with a Bio-Rad XRS system, and quantified with ImageQuant (Molecular Dynamics). For liposome tubulation assays, proteins were incubated with liposomes as above, after which they were spread onto glow-discharged, carbon-coated grids and stained with 5% uranyl acetate for analysis in the EM.

Radioligand Trafficking Assays

HeLa cells were transfected with siRNA duplexes for 72 hr as described above. For ^{125}I-transferrin trafficking, cells were washed into DMEM containing 25 mM Hepes, 0.2% fatty-acid-free BSA (DHB) and incubated at 37°C for 60 min with 1 kBq per well ^{125}I-transferrin to equilibriate the endosomal system. Cells were placed on ice and ^{125}I-transferrin remaining at the cell surface was stripped using ice cold 0.2 M acetic acid, 0.5 M NaCl (pH 4.5) for 2 min, then washed extensively with ice-cold PBS. Cells were chased into DHB containing 50 μg/ml cold transferrin for the indicated times. At the end of each time point, media was removed and separated into acid precipitable material (recycled counts) and acid soluble material (degraded counts) by incubation with 3% trichloroacetic acid, 0.3% phosphotungstic acid for 30 min at 4°C followed by high-speed centrifugation. A 2 min acid strip with 0.2 M acetic acid, 0.5 M NaCl, pH 2.8, removed transferrin bound to receptors at the cell surface. Cells were solubilized with 1 M NaOH at room temperature for 30 min. Fractions were subjected to γ counting and counts present in each fraction were determined by γ counting. Recycled counts were determined by summing acid precipitable counts and counts removed by the acetic acid strip. Degraded counts were deemed the acid soluble counts and counts released upon solubilisation of the cell monolayer were deemed internalized counts.

For ^{125}I-EGF trafficking assays, cells were washed into DHB and incubated at 4°C with 1 kBq per well ^{125}I-EGF for 1 hr. ^{125}I-bound to the cell surface was internalized by warming the cells to 37°C for 5 min. Cells were returned to ice and ^{125}I-EGF remaining at the cell surface was removed by a mild acid strip (0.2 M acetic acid, 0.5 M NaCl, pH 4.5) and washed extensively with ice cold PBS. Remaining cell surface receptors were saturated with 100 ng/ml EGF in DHB for 30 min at 4°C at which point cells were returned to 37°C for the chase time indicated. At the end of the chase, media was removed and a 2 min acid strip with 0.2M acetic acid, 0.5M NaCl, pH 2.8, removed ^{125}I-EGF bound to receptors at the cell surface. Cells were solubilized with 1M NaOH at room temperature for 30 min and recycled, degraded and internalized counts were collected as for the ^{125}I-transferrin trafficking assays.

Supplemental References

S1. Slot, J.W., Geuze, H.J., Gigengack, S., Lienhard, G.E., and James, D.E. (1991). Immuno-localization of the insulin regulatable glucose transporter in brown adipose tissue of the rat. J. Cell Biol. *113*, 123–135.

S2. Liou, W., Geuze, H.J., and Slot, J.W. (1996). Improving structural integrity of cryosections for immunogold labeling. Histochem. Cell Biol. *106*, 41–58.

S3. Mayer, L.D., Hope, M.J., and Cullis, P.R. (1986). Vesicles of variable sizes produced by a rapid extrusion procedure. Biochim. Biophys. Acta *858*, 161–168.