Developmental Cell *11*

Supplemental Data

Phosphatidylinositol 4-Phosphate Formation

at ER Exit Sites Regulates ER Export

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Supplemental Experimental Procedures

Materials:

GST, and GST fusion proteins (GST-PLCδ-PH, GST-PH-dynamin2, GST-PH-Grp1 (DNA provided by Dr. M. A. Lemmon, U. Penn.) GST-Fapp1-PH (DNA provided by Dr. T. Balla, NIH) GST-GFP-Fapp1-PH (referred to in the test as GFP-Fapp1-PH) and GST-Fapp1-PH^{NK} (W15N R18K) were produced in bacteria and purified using the bulk GST purification or the sarcosyl purification protocols (GST-Fapp1-PH^{NK}) according to manufacturer procedures (Phamacia Biotech). His-tagged Sac1 and Sac1 (C392S) were purified as described (Maehama et al., 2000); DNA provided by Dr. G.S. Taylor, U. Nebraska. Mutagenesis of GST-Fapp1-PH to generate GST-Fapp1-PH^{NK} was carried in two rounds using the Quickchange mutagenesis kit (Stratagene). Sar1-GTP (H79G), Sar1-GDP (T39N) and cytosolic Sec23/24 proteins were purified as described (Aridor et al., 1998). Sec13/31 complex was provided by Dr. W.E. Balch (TSRI). Purified proteins were dialyzed into buffer containing 25 mM Hepes pH 7.2 125 mM KOAc, which was supplemented with 1 mM $Mg(OAc)_2$ for Sar1 proteins. Protein concentration was determined using BioRad protein assay with BSA as a standard, and verified using a coomassie blue protein assay. Rat liver cytosol was prepared as described (Aridor et al., 1995). Antibodies to Sec23, Sec13, Sec12 and Sar1 and antibodies to VSV-G, Bet1 and GST were provided by Dr. W.E. Balch (TSRI). Antibody to Sec23 was also purchased from Affinity BioReagents (ABR). Antibody to GST was also purchased from Santa Cruz Biotechnology. Alexa 594 tagged anti-VSV-G Fab fragment (P5D4) was generated as described (Aridor et al., 2001). Anti-PtdIns4P was from Assay Designs, INC. HRP-conjugated anti-GST antibodies were from Sigma. Antibody to Mannosidase II was from Covance Research Product inc. Antibody to Giantin was provided by Dr. A. Linstedt, (Carnegie Mellon University, Pittsburgh PA). Secondary antibodies were from Pierce.

Methods:

Time-Lapse Laser-Scanning Confocal Microscopy

To visualize the dynamics of PtdIns4P formation, VSV infected cells were permeabilized, washed and incubated on ice for 20 min in the presence of Alexa 594-tagged Fab fragment of a monoclonal antibody (P5D4) to label VSV-G (Aridor et al., 2001). Labeled cells were washed and incubated in transport cocktail supplemented with rat liver cytosol

and GST-GFP-Fapp1-PH domain (0.2 μM) on a temperature controlled stage at 32°C to initiate ER export as described (Plutner et al., 1992). Oxyrase (0.3U/ml) (Oxyrase, Inc.) was added to the reaction to reduce photo damage. For dual wavelength time-lapse imaging, pair of fluorescence images were collected every 2 min using time-lapse confocal spinning video microscopy (Perkin Elmer, Ultraview). Excitation laserlines were 568 nm for Alexa 594 and 488 nm for GFP. Shutter and filter wheel timing and position were controlled by MetaMorph Software (Molecular Devices Corporation).

Subcellular Fractionation

ER and Golgi enriched membranes were obtained using a discontinuous gradient fractionation protocol (Balch et al., 1984a; Balch et al., 1984b). Briefly, crude liver homogenate (in 10 mM Tris pH 7.4, 5 mM EDTA 1 mM PMSF, 0.1 TIU/ml aprotinin, 5 μg/ml leupeptin) was centrifuged at 3000 rpm (Beckman J2-MC) for 10 min, supernatant and off-white upper pellet were collected and adjusted to 1.3 M sucrose (12 ml), placed on top of 2.4 M sucrose bottom (2 ml), and overlaid with 1.2 and 0.8 M sucrose (14 ml and 7 ml respectivaly) in a total volume of 35 ml. After centrifugation at 90000xg the membrane fractions at the interface between 0.8 and 1.2 M and the interface between 1.2 and 1.3 M sucrose were collected as fractions enriched in Golgi and ER membranes. The fractions were adjusted to 0.4 M sucrose and collected by centrifugation (100000xg 1hr at 4°C). Membrane fractions were tested for the enrichment of GM130 (*cis*-Golgi marker), Sec12 (ER marker) and for their ability to recruit Sec23 (Fig. S1).

Liposome Preparation and Lipid Binding Assay

Lipids were dried from chloroform stock solutions under a stream of nitrogen, hydrated in 20 mM Hepes pH 7.4, 0.25 M sorbitol and sonicated for 10 min in a solid state Ultrasonic FS-14 as previously described (Zhu et al., 1999). The final concentration of liposomes utilized in budding assays to neutralize Fapp-1-PH inhibition was 420μM PC/50μM PtdIns4P. Fapp1-PH^{WT} and Fapp1-PH^{NK} binding to PtdIns4P containing liposomes was performed as described (Drake et al., 2000) using liposomes composed of 10% Cholesterol, 40% PE, 40% PC and 10% PS or 10%, Cholesterol, 35% PE, 35% PC, 10% PS and 10% PtdIns4P in 10 mM Hepes pH 7.6, supplemented with 1 mM EDTA. Protein-Lipid overlay assays were performed as described (Dowler et al., 2000).

Coat Recruitment Assay

Sec23 recruitment assays were performed as described (Aridor and Balch, 2000; Aridor et al., 1995; Aridor et al., 1998), using 20-40 μg microsome membranes either unwashed or salt washed with 2.5 M urea, 30 min, on ice as indicated in figure legend. Membranes were incubated with Sar1-GTP (0.25 μg) and Sec23/24 (0.□ μg) or with Sar1-GTP (0.1 μg) and RLC (50 μg), with or without Fapp1-PH domain in buffer containing 35 mM Hepes-KOH pH7.4, 2.5 mM $Mg(OAc)_2$, 80 mM KOAc, 250 mM sorbitol, 5 mM EGTA, 1.6 mM $CaCl_2$, 0.2 mM GTP, 0.1 mM GDP and either 1 mM ATP or 1 mM ATP, 5 mM creatine phosphate, 0.2 IU of rabbit muscle creatine phosphokinase as indicated, in a final volume of 60 μl for 15 min at 32°C followed by further 10 minutes incubation on ice. Subsequently membranes were washed in a buffer containing 25 mM Hepes pH 7.2, 2.5 mM $Mg(OAc)_2$ and 250 mM KOAc, and collected by centrifugation for SDS PAGE and western blot analysis. For ATP substitution experiments, the recruitment assay was performed using

0.3 μg Sar1-GTP and cytosol supplemented with 50 μM diC16 PtdIns4P (Echelon) as indicated. PtdIns4P micelles were prepared in 20 mM Hepes-KOH pH7.4, 250 mM sorbitol.

Quantitation of Sec23 and GST-Fapp1-PH Colocalization

For analysis of Sec23 and GST-Fapp1-PH co-localization, GST-Fapp1-PH was utilized at 3μM, a concentration that is sub-inhibitory to COPII coat assembly (not shown) yet is within the binding affinity of the domain to PtdIns4P containing liposomes. Single optical sections taken at 60X were collected using an Olympus Fluoview 500 scanning confocal microscope (Olympus America). Metamorph (Molecular Devices Corporation) was used to count the number of labeled structures inside the cell using background fluorescence as a cell reference. The number of GST-Fapp1-PH and Sec23 positive structures was counted automatically using the segmented cells as defined regions. To define individual red and green structures inside the cell the program was trained to recognize the structures as thresholded objects of defined size. To quantify co-localization, a binary mask was generated for each color. Overlapping structures were defined using a Boolean AND statement. Subsequently the number of co-localized structures was counted using the same algorithm as defined for quantifying individually labeled structures. In this analysis, the perinuclear region of the cells was excluded because of the high density of Golgi elements positive for Fapp1-PH that are located in close proximity to ERES.

Quantitation of VSV-G^{ts} Export from ER to VTCs

Confocal scanning microscopy was performed using Olympus FluoView 500 microscope (Olympus America) with 60X, 1.4 NA planapochromat oil-immersion objective (Olympus America). Optical sections of 0.2 μm were acquired using FluoView Software(Olympus, America). Individual experiments were performed with identical laser output levels, exposure times, and scaling. Single optical sections of fields, each containing 20-40 cells, were taken for analysis. Transport of VSV-G^{ts} was assessed by appearance of VSV-G in bright punctate structures. MetaMorph software (Molecular Devices Corporation) was utilized to count the number of punctate structures in the cell using background fluorescence as a cell reference and applying a low pass smoothing filter. Fixed inclusive threshold was then manually chosen for VSV-G exporting sample, and was applied to all images. Subsequently, the numbers of VTCs (bright puctate structures) were calculated automatically. The number of cells per field was determined manually.

We also analyzed the mobilization of VSV-G^{ts} to VTCs using de-convolved projections created from five consecutive 0.2μm optical sections. Images stacks consisting of optical slices taken 0.2 μm apart were de-convolved using a constrained iterative algorithm in SlideBook software. Threshold segmentation was used to create a mask for VTCs in five representative fields, each containing 4-15 cells. Values used for segmentation were derived from controls and used for all samples. This analysis yielded essentially similar results.

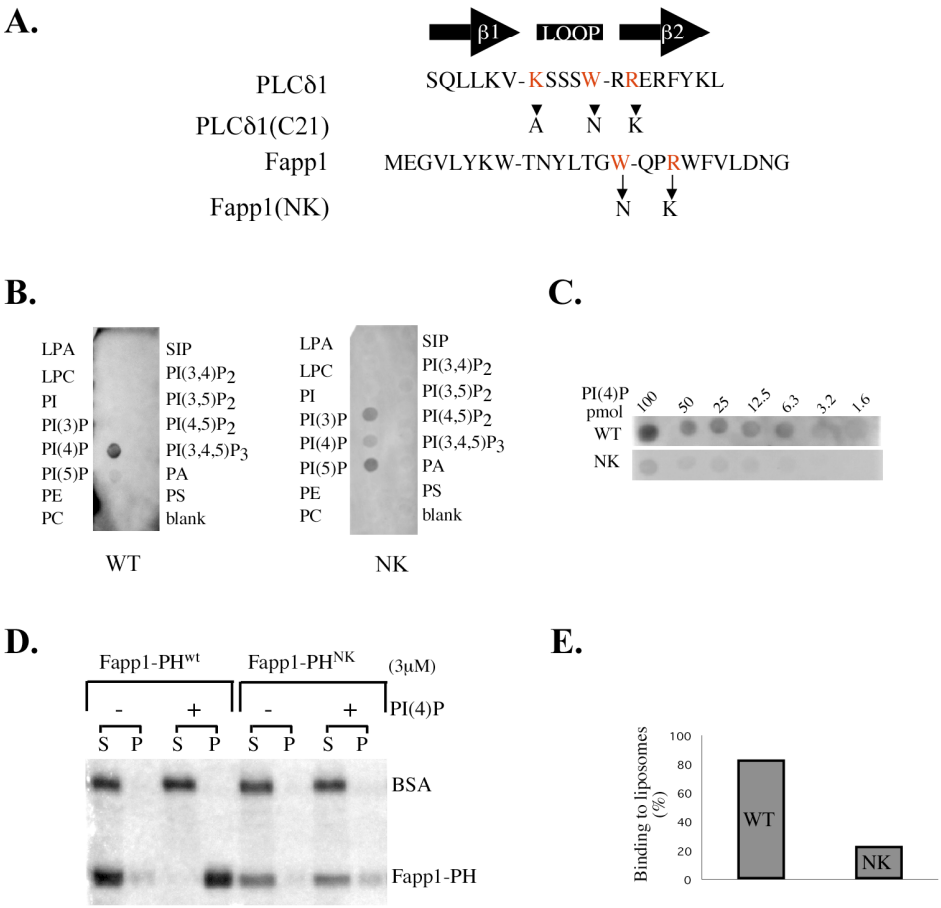

Figure S1. Lipid-Binding Properties of WT and Mutated Fapp1-PH Domains

A. Alignment of PtdInts binding motifs (β1 strand β1β2 loop and β2 strand) of PLCδ1-PH and Fapp1-PH. Amino acids are presented using a single-letter code. Mutated amino acids previously characterized for PLCδ1-PH (C21) (Jost et al., 1998) and introduced in Fapp1-PH^{NK} are shown in red. Arrows point to amino acids that were substituted. **B.** GST-Fapp1-PH^{WT} or GST-Fapp1-PH^{NK} (1 μg/ml) were incubated with membrane strips on which phospholipids (100 pmol) were immobilized as indicated. The membranes were washed and bound Fapp1 proteins were detected with anti-GST antibody conjugated to HRP. **C.** PtdIns4P membrane arrays on which indicated amounts of PtdIns4P were immobilized, were incubated with GST-Fapp1-PH^{WT} or GST-Fapp1-PH^{NK} (1 μg/ml). The membranes were washed and bound Fapp1 domains were detected with anti GST antibody conjugated to HRP. **D.** Fapp1-PH^{wt} or Fapp1-PH^{NK} (3 μM), were incubated with control or PtdIns4P containing liposomes as described in Materials and Methods. At the end of incubation, the liposomes were collected by centrifugation. Aliquots corresponding to equal amounts of supernatant (S) and of pellet (P) were resolved on SDS–PAGE gels and stained with Coomassie Blue. The localization of BSA (added to the reaction as a marker that remains soluble in the supernatant) and of the GST-Fapp1-PH domains is indicated **E.** Quantitation of D. Similar results were obtained at least in two independent experiments.

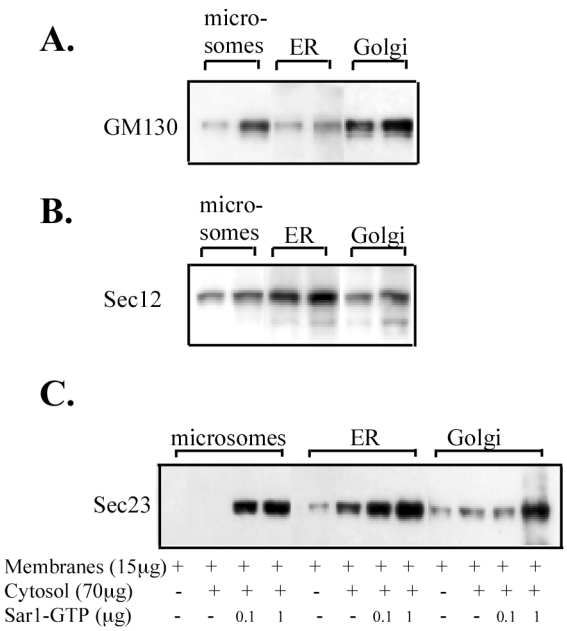

Figure S2. Fractionation of Rat Liver Membranes to Enrich for ERES

NRK microsomes, (loaded at 5 μg and 10 μg), ER enriched rat liver membranes (5 μg and 10 μg, collected from the 1.2 to 1.4 interface of a discontinuous sucrose density gradient as described in supplement Materials and Methods) or Golgi enriched membranes (5 μg and 10 μg, collected from the 0.8 to 1.2 interface) were analyzed for **(A)** GM130 (Golgi marker) or **(B)** Sec12 (ER marker) **C.** Microsomes, ER and Golgi enriched membranes were tested for their ability to recruit Sec23. Indicated membranes were incubated with RLC at 32^{o}C for 15 min in the presence or absence of Sar1-GTP as indicated. The recruitment of Sec23 to the membranes was determined by western blot.

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