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Supplemental materials and methods

Reagents

Amphotericin B, ATPγS, Brij 30, Brij 35, Brij 72, Brij 96V, CHAPS, cholic acid (sodium salt), ergosterol, filipin III, GppNHp, GTP, GTPγS, n-octyl-β-D-glucopyranoside, nystatin, Triton X-100, Tween 20, and monoclonal anti-ceramide antibody were purchased from Sigma-Aldrich. PI(4)P, PI(4,5)P_2, PIP-Strips, and mAbs to PI(3)P, PI(3,4)P_2, and PI(3,5)P_2 were purchased from Echelon Biosciences. mAbs to PI(4,5)P_2 and PI(3,4,5)P_3 and Alexa Fluor 488 signal-amplification kit for fluorescein-conjugated probes were purchased from Molecular Probes. Monoclonal anti-phosphatidylserine antibody was purchased from Upstate Biotechnology. Fluorescein-conjugated goat anti–mouse IgG antibodies and fluorescein-conjugated goat anti–mouse IgM antibodies were obtained from Jackson ImmunoResearch Laboratories. *L*-[^{35}S]methionine was purchased from MP Biomedicals. Guanosine 5′-α-[^{32}P]triphosphate was obtained from Amersham Biosciences. N-palmitoyl-D-*erythro*-sphingosine (ceramide), ceramide-1-phosphocholine (sphingomyelin), D-*erythro*-sphingosine, 1,2-dioleoyl-*sn*-glycerol (diacylglycerol), 1,2-dioleoyl-*sn*-glycero-3-phosphate (phosphatidic acid), 1,2-dioleoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (phosphatidylglycerol), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (phosphatidylcholine), 1-oleoyl-2-hydroxy-*sn*-glycero-3-phosphate (lysophosphosphatidic acid), 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (phosphatidylethanolamine), L-α-phosphatidylinositol (phosphatidylinositol), and 1,2-dioleoyl-*sn*-glycero-3-[phospho-L-serine] (phosphatidylserine) were obtained from Avanti Polar Lipids, Inc.

In vitro assay for peroxisome docking

P1 and P2 vesicles were individually preprimed by incubation in PF buffer (15 mM MES, pH 6.0, 100 mM KCl, 50 mM KOAc, 3 mM $MgCl_2$, and 2 mM MgOAc) containing 250 mM sorbitol, 1 mg cytosol/ml, 1 mM ATP, 40 mM creatine phosphate, and 10 U creatine kinase/ml. Cytosol was prepared in ice-cold PF buffer (Rexach and Schekman, 1991). After incubation for 10 min at 26°C, peroxisomal vesicles were pelleted by centrifugation at 100,000 *g* for 5 min at 4°C in a TLA120.2 rotor, resuspended in ice-cold PF buffer containing 250 mM sorbitol, and repelleted. Peroxisomal vesicles were then resuspended in PF buffer supplemented with 250 mM sorbitol and incubated individually with or without 300 μg nystatin/ml, 80 μM phosphoinositide-specific mAbs, 2 mM GTPγS or 1 mM ATPγS, in the presence or absence of 1 mg cytosol/ml, as indicated. After incubation for 5 min at 26°C, P1 and P2 were mixed and supplemented with 1 mM ATP, 40 mM creatine phosphate, and 10 U creatine kinase/ml. After a 10-min incubation at 26°C, a 80-μl sample of peroxisomal vesicles was transferred to the bottom of a 5-ml ultraclear centrifuge tube (Beckman Coulter) and supplemented with four volumes of 62.5% (wt/wt) sucrose in ice-cold PF buffer in order to adjust the sucrose concentration of the sample to 50% (wt/wt). The sample was overlaid with 500 μl of 30% sucrose, 600 μl of 28% sucrose, 1 ml of 26% sucrose, 1 ml of 24% sucrose, 1 ml of 22% sucrose, and 500 μl of 10% sucrose (all wt/wt in PF buffer). After centrifugation at 200,000 *g* for 18 h at 4°C in a SW50.1 rotor (Beckman Coulter), nine fractions of 555 μl each were collected. Proteins recovered in equal volumes of gradient fractions were precipitated by adding six volumes of acetone, resolved by SDS-PAGE, and then visualized by immunoblotting with anti-Pex1p, anti-Pex16p, and anti-thiolase antibodies. Equal volumes of gradient fractions were also subjected to the protein-lipid overlay assay, GTP slot-blot, lipid extraction, and TLC.

In vitro peroxisome fusion assay

P1 and P2 vesicles were preincubated individually in PF buffer containing 250 mM sorbitol for 5 min at 26°C without inhibitor or with various amounts of ergosterol ligands, phosphoinositide-specific antibodies, or nonhydrolyzable GTP analogues. Pretreated P1 and P2 were then mixed and supplemented with 1 mg cytosol/ml, 1 mM ATP, 40 mM creatine phosphate, and 10 U creatine kinase/ml to yield standard fusion reactions. Cytosol was prepared in ice-cold PF buffer (Rexach and Schekman, 1991). After a 60-min incubation at 26°C, individual samples were analyzed by immunoblotting with antibodies to the peroxisomal matrix protein thiolase (THI). These antibodies detected both the precursor form (pTHI) of THI and its mature form (mTHI) (Titorenko et al., 2000). Antigen-antibody complexes were detected by ECL Western Blotting Detection Reagents (Amersham Biosciences). Digital images were acquired using the VersaDoc Imaging System (Bio-Rad Laboratories). The data were analyzed with the Discovery Series Quantity One 1-D Analysis Software (Bio-Rad Laboratories). The extent of proteolytic processing of pTHI to mTHI, which is directly proportional to the number of fused peroxisomes (Titorenko et al., 2000), was used to quantitate peroxisome fusion.

In vitro assay of association of Pex1p and Pex6p with P1 and P2

L-[^{35}S]methionine-labeled P1 and P2 were preincubated individually in PF buffer containing 250 mM sorbitol for 5 min at 26°C with or without 300 μg nystatin/ml, 300 μg filipin III/ml, 300 μg amphotericin B/ml, 80 μM phosphoinositide-specific mAbs, 1 mM ATPγS, 2 mM GTPγS, or 3 mM GppNHp, in the presence or absence of 1 mg cytosol/ml, as indicated. Pretreated P1 and P2 were then supplemented with 1 mM ATP, 40 mM creatine phosphate, and 10 U creatine kinase/ml, and incubated individually in the presence or absence of 1 mg of unlabeled cytosol/ml, as indicated. After a 10-min incubation at 26°C, peroxisomal vesicles were pelleted by centrifugation at 100,000 *g* for 5 min at 4°C in a TLA120.2 rotor. Proteins from the pellet and supernatant fractions were subjected to immunoaffinity chromatography under denaturing conditions with anti-Pex1p or anti-Pex6p antibodies linked to protein A–Sepharose.

Immunoaffinity chromatography

Proteins that were recovered in the pellet and supernatant fractions in the in vitro assay of association of Pex1p and Pex6p with P1 and P2 were subjected to immunoaffinity chromatography under denaturing conditions. Anti-Pex1p or anti-Pex6p antibodies were covalently linked to protein A–Sepharose as described previously (Xu et al., 1998). Samples in PF buffer containing 250 mM sorbitol were diluted with an equal volume of 4% SDS and warmed at 65°C for 10 min. Samples were then allowed to cool to RT, and four volumes of 62.5 mM Tris-HCl, pH 7.5, buffer containing 190 mM NaCl, 1.25% (vol/vol) Triton X-100, and 6 mM EDTA were added. Samples were cleared of any nonspecifically binding proteins by incubation for 20 min at 4°C with protein A–Sepharose washed five times with 10 mM Tris-HCl, pH 7.5. The cleared samples were subjected to immunoaffinity chromatography. Bound proteins were washed five times with 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% (vol/vol) Triton X-100, and eluted with 2% SDS at 95°C for 5 min. Eluted proteins were resolved by SDS-PAGE and visualized by fluorography (Titorenko et al., 1998).

Osmotic lysis of peroxisomes

Purified P1 and P2 vesicles were lysed by incubation on ice for 20 min in LC buffer (20 mM Hepes-KOH, pH 8.0, and 50 mM NaCl).

EM

Detergent-resistant ECR domains floated to low density during centrifugation in a sucrose density gradient and peaked in fraction 7 of the gradient. A 200-μl aliquot of purified ECR domains in MBS buffer (25 mM MES/KOH, pH 6.5, and 150 mM NaCl) was mixed with 400 μl of ice-cold 150 mM sodium cacodylate buffer, pH 7.2, containing 3% glutaraldehyde. Immediately after mixing the sample and glutaraldehyde solution, 600 μl of 2% OsO_4 in ice-cold CD buffer (100 mM sodium cacodylate, pH 7.2) was added. After a 2-h incubation on ice, fixed ECR domains were sedimented at 100,000 *g* for

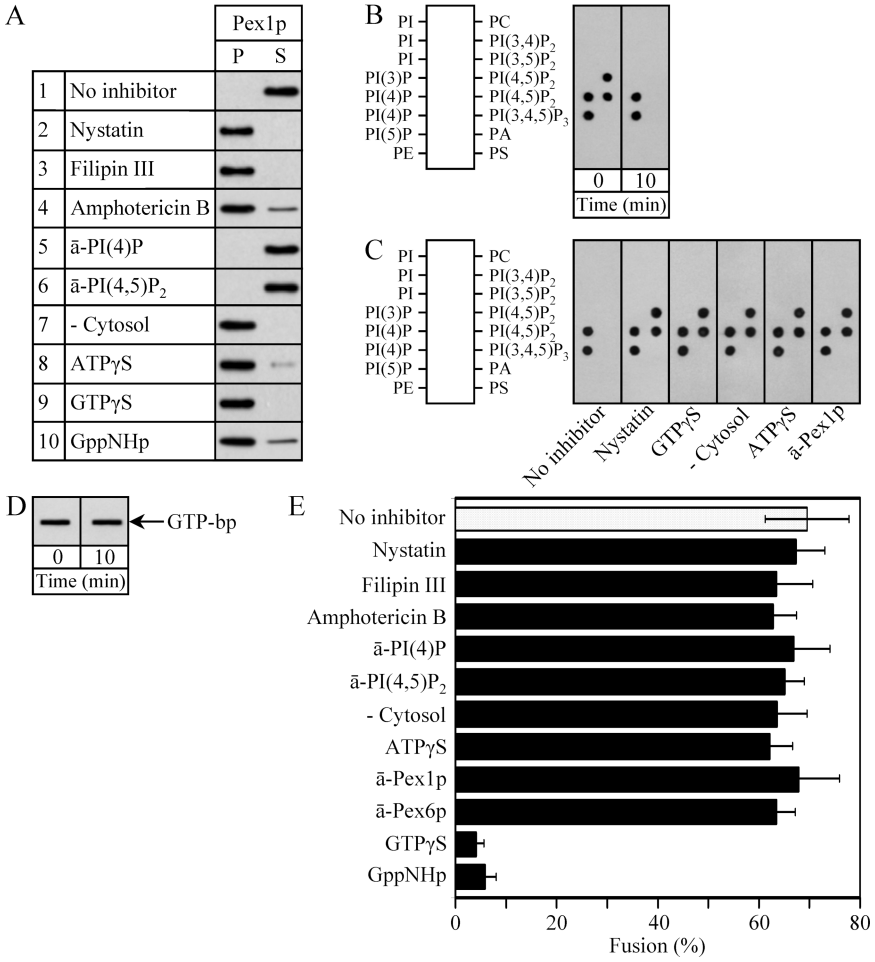


Figure S1. **Dynamics of the association of Pex1p, phosphoinositide-, and GTP-binding proteins with membranes of primed P1 and P2 during their docking and the requirements for the fusion of docked P1 and P2.** (A) P1 and P2 were individually preprimed by incubation with cytosol and ATP for 10 min at 26°C. Peroxisomal vesicles were reisolated by centrifugation, washed, and resuspended in buffer. Reisolated P1 and P2 were incubated individually for 5 min at 26°C with or without ergosterol ligands, phosphoinositide-specific antibodies, ATPγS, or nonhydrolyzable GTP analogues in the presence or absence of cytosol, as indicated. P1 and P2 were then mixed and supplemented with ATP. After a 10-min incubation at 26°C, peroxisomal vesicles were subjected to centrifugation to yield supernatant (S) and pellet (P) fractions. Recovered proteins were resolved by SDS-PAGE and immunoblotted with antibodies to Pex1p. (B and D) Intact P1 and P2, either labeled with *L*-[^{35}S]methionine (B) or unlabeled (D), were primed individually by incubation with cytosol and ATP for 10 min at 26°C. Peroxisomal vesicles were then reisolated by centrifugation, washed, and resuspended in buffer. Preprimed P1 and P2 were mixed and supplemented with ATP. After a 10-min incubation at 26°C, peroxisomal vesicles were osmotically lysed and subjected to centrifugation to yield supernatant (matrix proteins) and pellet (membrane proteins) fractions. Pelleted membrane proteins were solubilized with a detergent, n-OG. Detergent-soluble membrane proteins were analyzed by protein-lipid overlay assay using commercial PIP-Strips (B) or by GTP slot-blot with guanosine 5′-α-[^{32}P]triphosphate (D). Lipid- and GTP-binding proteins were visualized by autoradiography. (C) *L*-[^{35}S]methionine–labeled P1 and P2 were individually preprimed by incubation with cytosol and ATP for 10 min at 26°C. Peroxisomal vesicles were then reisolated by centrifugation, washed, and resuspended in buffer. Reisolated P1 and P2 were incubated individually for 5 min at 26°C with or without nystatin, GTPγS, ATPγS, or anti-Pex1p antibodies in the presence or absence of cytosol, as indicated. P1 and P2 were then mixed and supplemented with ATP. After a 10-min incubation at 26°C, peroxisomal vesicles were osmotically lysed and subjected to centrifugation to yield supernatant (matrix proteins) and pellet (membrane proteins) fractions. Pelleted membrane proteins were solubilized with n-OG. Detergent-soluble membrane proteins were analyzed by protein-lipid overlay assay using commercial PIP-Strips. Lipid-binding proteins were visualized by autoradiography. (E) The docking complex P1/P2 was purified by flotation on a multistep sucrose gradient (Titorenko et al., 2000). This complex was incubated for 60 min at 26°C with or without ergosterol ligands, phosphoinositide-specific antibodies, ATPγS, antibodies to Pex1p or Pex6p, or nonhydrolyzable GTP analogues in the presence or absence of cytosol, as indicated. The efficiency of membrane fusion was monitored by the extent of proteolytic processing of the 47-kD precursor form (pTHI) of thiolase (THI) to the 45-kD mature form (mTHI) of this peroxisomal matrix protein (Titorenko et al., 2000).

20 min at 4°C in a TLS*55* rotor (Beckman Coulter) onto a bed (25–50 μl) of hardened, low-melting 2.5% NuSieve GTG agarose (FMC). The pellet was postfixed in a solution of 1% OsO_4 plus 2.5% $K_2Cr_2O_7$ in ice-cold CD buffer for 2 h on ice. The pellet was rinsed twice with ice-cold CD buffer and exposed to 0.05% tannic acid in the same buffer. After a 30-min incubation on ice, the pellet was washed once with ice-cold CD buffer and three times with water. The pellet was incubated overnight with 2% uranyl acetate in water at 4°C and then washed three times with water. After dehydration in a graded ethanol series, the fixed and stained sample was embedded in Poly/Bed 812 epoxy resin (Polysciences). Silver/gold thin sections from the embedded blocks were examined in a transmission electron microscope (model JEM-2000FX; JEOL).

Preparation of ergosterol-containing liposomes

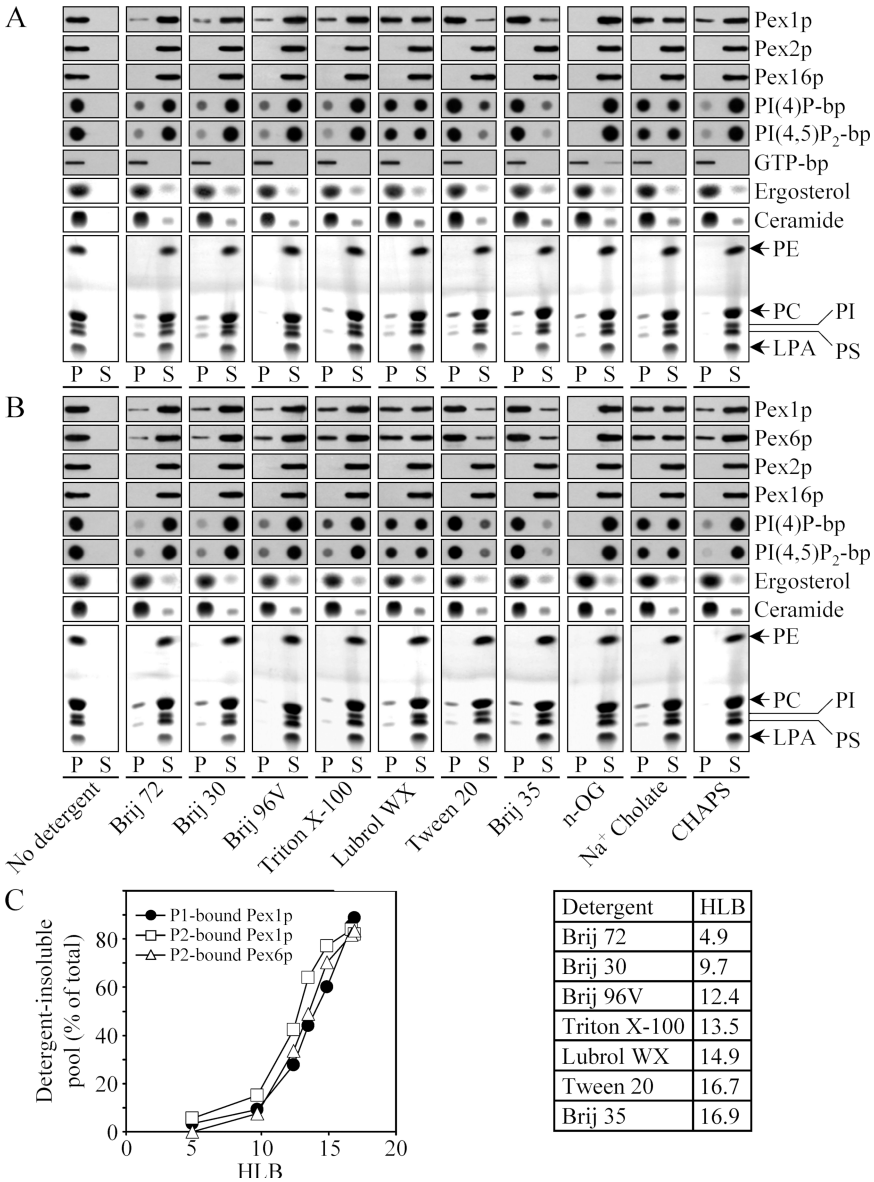
Uniformly sized, unilamellar liposomes were made of PC containing or not containing ergosterol (3:1 weight ratio), as described previously (MacDonald et al., 1991), using LiposoFast Liposome "Factory" (Avestin). The liposomes were formed by extrusion through polycarbonate membranes with a pore diameter of 100 nm, according to the manufacturer's instructions.

Detergent treatment of peroxisomal membranes

The pellet of membranes recovered after centrifugation of osmotically lysed P1 or P2 was resuspended in ice-cold MBS buffer (25 mM MES-KOH, pH 6.5, and 150 mM NaCl) to a final concentration of 2 mg/ml. Equal aliquots of the suspension of membranes were supplemented with one of the following detergents: Brij 30, Brij 35, Brij 72, Brij 96V, CHAPS, cholic acid (sodium salt), n-octyl-β-D-glucopyranoside (n-OG), Triton X-100, and Tween 20. 5 mg of detergent was used for the treatment of 1 mg of peroxisomal membrane protein. After incubation on ice for 30 min, samples were subjected to centrifugation at 100,000 *g* for 30 min at 4°C. Equal portions of the pellet and supernatant fractions were analyzed by SDS-PAGE, followed by immunoblotting or silver staining. Equal portions of both fractions were also subjected to protein-lipid overlay assays, GTP slot-blot, lipid extraction, and TLC.

Protein-lipid overlay assays

All protein-lipid overlay assays were conducted with membrane proteins of P1 and P2 vesicles that were purified from *L*-[^{35}S]methionine–labeled cells. To evaluate the phospholipid-binding specificity of these proteins, the pellet of membranes recovered after centrifugation of osmotically lysed P1 or P2 was resus-



Detergent	HLB
Brij 72	4.9
Brij 30	9.7
Brij 96V	12.4
Triton X-100	13.5
Lubrol WX	14.9
Tween 20	16.7
Brij 35	16.9

Figure S2. **Effect of various detergents on the solubility of proteins and lipids associated with the membranes of unprimed P1 and P2.** (A and B) The pellet of membranes recovered after centrifugation of osmotically lysed unprimed P1 (A) or P2 (B) was resuspended in ice-cold MBS buffer. Equal aliquots of the suspension of membranes were exposed to various detergents as described in the supplemental Materials and methods. After a 30-min incubation on ice, samples were subjected to centrifugation at 100,000 *g* for 30 min at 4°C to yield pellet (P; detergent-insoluble) and supernatant (S; detergent-soluble) fractions. Proteins from equal portions of the pellet and supernatant fractions were immunoblotted with the indicated antibodies. Equal portions of the pellet and supernatant fractions were also subjected to protein-lipid overlay assays using nitrocellulose membrane arrays spotted with PI(4)P or PI(4,5)P_2, GTP slot-blot, and lipid extraction, which was followed by TLC and visualization of lipids. (C) The extent of insolubility of P1- and P2-bound Pex1p and Pex6p in various nonionic detergents of the polyoxyethylene group was quantitated by densitometric analysis of immunoblots presented in A and B. The values of hydrophilic-lipophilic balance (HLB) for the indicated detergents are from Röper et al. (2000).

pended in buffer TBSO (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% n-OG) and incubated for 30 min on ice. Samples were subjected to centrifugation at 100,000 *g* for 30 min at 4°C. Under these conditions, n-OG completely solubilized the vast majority of all membrane proteins (Fig. S3). The supernatants of n-OG–solubilized proteins were incubated at 5 μg/ml with the PIP-Strip (Echelon Biosciences) at 4°C overnight. After washing the PIP-Strip five times for 5 min each with TBSO, autoradiography was used to detect binding of membrane proteins to phospholipids.

To evaluate the solubility of PI(4)P- and PI(4,5)P_2-binding proteins in various detergents, nitrocellulose membrane arrays were spotted with 100 pmol of PI(4)P or PI(4,5)P_2. Membranes were cut into pieces, each containing a single phosphoinositide spot, and used for protein-lipid overlay assays. The pellet of membranes recovered after centrifugation of osmotically lysed P1 or P2 was resuspended in buffer TBS (10 mM Tris-HCl, pH 8.0, 150 mM NaCl) containing either no detergent or the detergent of interest. After incubation for 30 min on ice, samples were subjected to centrifugation at 100,000 *g* for 30 min at 4°C. The supernatants of detergent-soluble membrane proteins were processed for protein-lipid overlay assays as described above. The pellets of detergent-insoluble membrane proteins were washed with TBS and resuspended in buffer TBSO containing n-OG, a detergent that completely solubilized the vast majority of all membrane proteins (Fig. S3). After incubation for 30 min on ice, samples were subjected to centrifugation at 100,000 *g* for 30 min at 4°C. The supernatants of n-OG-solubilized proteins were then processed for protein-lipid overlay assays as described above.

To evaluate the levels of PI(4)P- and PI(4,5)P_2-binding proteins in fractions of flotation gradients that were used for the fractionation of Brij 35–treated membranes of P1 and P2, nitrocellulose membrane arrays were spotted with 100 pmol of PI(4)P or PI(4,5)P_2. These nitrocellulose membranes were cut into pieces, each containing a single phosphoinositide spot, and used for protein-lipid overlay assays. After being diluted with nine volumes of TBSO buffer, equal volumes of flotation gradient fractions were processed for the overlay assays as described above.

Lipid analyses

For extraction of lipids, 1.2 ml of chloroform/methanol (1:1, vol/vol) were added to each of the 555-μl fractions recovered in the sucrose density gradient for isolation of ergosterol- and ceramide-rich membrane domains. After incubation on ice for 15 min, samples were subjected to centrifugation at 20,000 *g* for 15 min at 4°C. The chloroform phase was separated and dried under nitrogen. The lipid film was dissolved in 60 μl of chloroform (for the analysis of ergosterol and ceramide) or 60 μl of chloroform/methanol (1:1, vol/vol) (for the analysis of glycerophospholipids). 20 μl of each sample were spotted on 60 Å silica gel plates for TLC (Whatman). The lipids were developed in the following solvent systems: chloroform/acetone (4:1, vol/vol) (for the analysis of ergosterol and ceramide) and chloroform/methanol/water (65:25:4, vol/vol) (for the analysis of glycerophospholipids). All lipids were detected using 5% phosphomo-

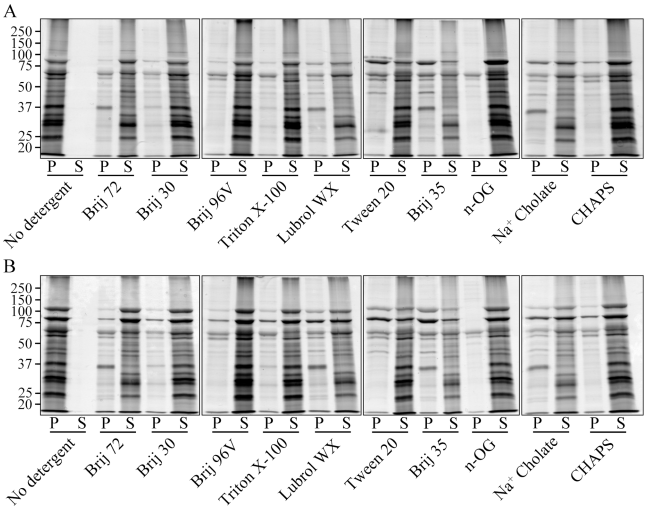


Figure S3. **Spectra of detergent-soluble and -insoluble membrane proteins of the immature peroxisomal vesicles P1 and P2.** Unprimed P1 (A) or P2 (B) vesicles were osmotically lysed and subjected to centrifugation at 4°C to yield supernatant (matrix proteins) and pellet (peroxisomal membranes) fractions. Pelleted membranes were resuspended in ice-cold MBS buffer. Equal aliquots of the suspension of membranes were then exposed to various detergents as described in supplemental Materials and methods. The samples were subjected to centrifugation at 100,000 *g* for 30 min at 4°C to yield pellet (P; detergent-insoluble) and supernatant (S; detergent-soluble) fractions. Proteins from equal portions of the pellet and supernatant fractions were analyzed by SDS-PAGE and silver staining.

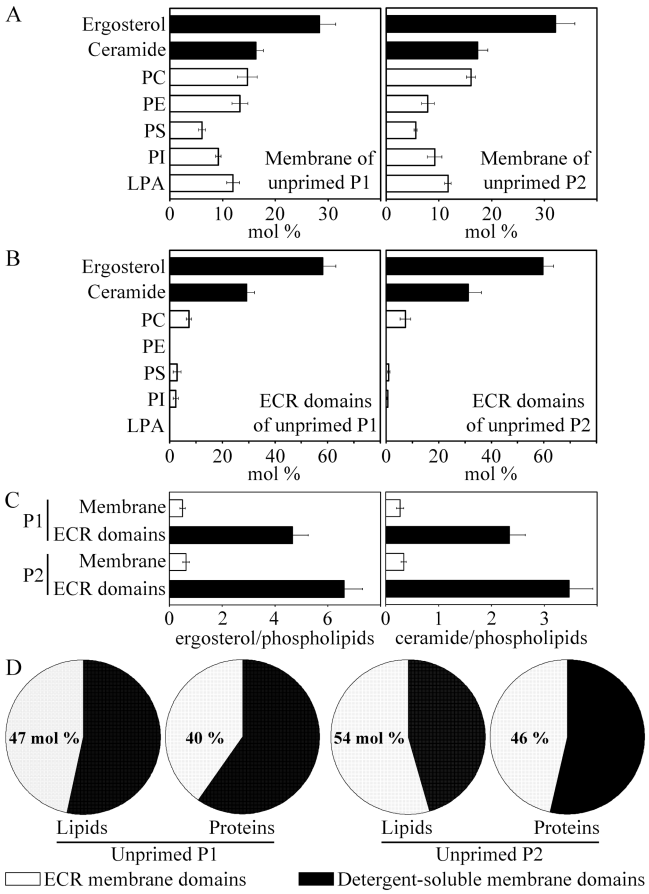


Figure S4. **Lipid composition of the membranes of unprimed P1 and P2 and of their ECR domains.** (A) Lipid concentrations in the total membranes of P1 and P2 vesicles. Lipids extracted from membranes of unprimed P1 and P2 were analyzed by TLC and quantitated as described in the supplemental Materials and methods. Concentrations of individual lipid species are presented in mole percent (mol %). (B) Lipid concentrations in ECR domains of membranes of P1 and P2 vesicles. Lipids from the TLC plates shown in Fig. 4 were quantitated. The mole percent concentrations of individual lipid species recovered in the low-density fractions 5 to 9 of the flotation gradients are shown. (C) Ergosterol/total glycerophospholipids and ceramide/total glycerophospholipids ratios for ECR domains were calculated from the data presented in B. (D) ECR domains represent a substantial fraction of the membranes of unprimed P1 and P2. Lipids and proteins in fractions of the flotation gradients shown in Fig. 4 were quantitated. Percent recoveries of lipids and proteins in ECR domains and in detergent-soluble portions of the membranes of P1 and P2 are presented.

lybdic acid in ethanol and visualized by heating for 30 min at 110°C. Lipids were quantitated by densitometric analysis of TLC plates as described previously (Fried and Sherma, 1999), using lipid standards in the 0.1–0.5 μg range for calibration.

To evaluate the distribution of ceramide and PS between the two leaflets of the membrane bilayers of P1 and P2 vesicles, the suspension of purified P1 or P2 in ice-cold buffer H (10 mM MES-KOH, pH 5.5, 250 mM sorbitol, 1 mM KCl, 0.5 mM EDTA, and 1× protease inhibitor cocktail; Titorenko et al., 1998) at 1 mg of protein/ml was divided into two equal aliquots. One aliquot remained untreated, whereas peroxisomal vesicles in the other aliquot were lysed by incubation on ice for 20 min in 20 mM Hepes-KOH buffer, pH 8.0, containing 50 mM NaCl and 1× protease inhibitor cocktail (Guo et al., 2003). The pellet of membranes recovered after centrifugation of osmotically lysed P1 or P2 was resuspended in ice-cold buffer H at 1 mg of protein/ml. Twofold serial dilutions of intact P1 or P2 vesicles (from the first aliquot) and of the membranes recovered after osmotic lysis of these vesicles (from the second aliquot) in the range of 10–160 μg of protein/ml were made in ice-cold buffer H. Anti-ceramide mouse IgG or anti-PS mouse IgM were added to concentrations 0.5 and 1 μg/ml, respectively. After incubation for 30 min on ice, samples were subjected to centrifugation at 100,000 *g* for 8 min at 4°C. The pellets were resuspended in 200 μl of ice-cold buffer H and supplemented with fluorescein-conjugated goat anti–mouse IgG or fluorescein-conjugated goat anti–mouse IgM antibodies at 2.5 and 5 μg/ml, respectively. After incubation for 30 min on ice, samples were subjected to centrifugation at 100,000 *g* for 8 min at 4°C. The pellets were resuspended in 200 μl of ice-cold buffer H and supplemented with Alexa Fluor 488 rabbit anti-fluorescein/Oregon green IgG at 15 μg/ml. After incubation for 30 min on ice, samples were subjected to centrifugation at 100,000 *g* for 8 min at 4°C. The pellets were resuspended in 200 μl of ice-cold buffer H and supplemented with Alexa Fluor 488 goat anti–rabbit IgG at 20 μg/ml. After incubation for 30 min on ice, samples were subjected to centrifugation at 100,000 *g* for 8 min at 4°C. The pellets were resuspended in 200 μl of ice-cold buffer H and placed into the wells of a 96-well microplate. The fluorescence of samples was measured using the Wallac Victor 2 Multi-label microplate fluorescence reader with filters set at 485 (± 7.5) nm (excitation) and 510 (± 5) nm (emission). Controls were made for each dilution of intact P1 and P2 and of peroxisomal membranes recovered after osmotic lysis of these peroxisomal vesicles. The controls included normal mouse IgG or IgM at 0.5 and 1 μg/ml, respectively, added instead of anti-ceramide mouse IgG or anti-PS mouse IgM. Background fluorescence, which was due to the nonspecific binding of mouse IgG, mouse IgM, and/or fluorescein- or Alexa Fluor 488–labeled antibodies to the peroxisomal membrane, was subtracted.

Mass spectrometry

Proteins were resolved by SDS-PAGE and visualized by silver staining (Shevchenko et al., 1996). Protein bands were excised from the gel, reduced, alkylated, and in-gel digested with trypsin (Shevchenko et al., 1996). The proteins were identified by matrix-assisted laser desorption/ionization mass spectrometric peptide mapping (Jiménez et al., 1998) using a Micromass M@LDI time-of-flight mass spectrometer (Waters). Database searching using peptide masses was performed with the Mascot web-based search engine. The identification of Pex1p by mass spectrometric peptide mapping was based on the analysis of 12 peptides of a 100-kD protein. These peptides covered 15.4% of the Pex1p sequence with mass accuracy better than 100 ppm over the mass to charge ratio range of 700 to 2400. 10 peptides of a 116-kD protein that were used for the identification of Pex6p covered 14.1% of the protein sequence with mass accuracy better than 100 ppm over the mass to charge ratio range of 1000 to 2400.

A two-stage assay for defining the hierarchy of membrane-associated events during peroxisome priming and docking

To define the hierarchy of ergosterol-, PI(4)P-, PI(4,5)P_2-, GTP hydrolysis-, cytosol-, and ATP hydrolysis-dependent steps during the priming- and docking-specific remodeling of the membrane-associated peroxisome fusion machinery, we developed a two-stage assay. This assay examines if the step affected by one inhibitor precedes, occurs in parallel, or follows the step sensitive to another inhibitor. For example, in the two-stage assay for the priming-specific movement of P1-bound Pex1p and of P2-associated Pex6p from detergent-resistant ECR domains to detergent-soluble, ergosterol- and ceramide-poor membrane domains, the fusion partners P1 and P2 are initially (the first stage) incubated individually with inhibitor 1. Separately pretreated P1 and P2 vesicles are then reisolated by centrifugation and washed in buffer. During the second stage, pretreated and washed peroxisomal vesicles are supplemented with inhibitor 2 and the compound that overcomes the block imposed by inhibitor 1. After incubation, the effect of the sequential treatment with inhibitors on the monitored event, i.e., the relocation of P1-bound Pex1p and of P2-associated Pex6p from ECR domains to an ergosterol- and ceramide-poor portion of the peroxisomal membrane, is assessed. To evaluate the efficiency of the monitored event, Brij 35–treated membranes of P1 and P2 are subjected to centrifugation by flotation in a discontinuous sucrose density gradient as described in Materials and methods, and the recovery of Pex1p or Pex6p in gradient fractions is examined by immunoblotting. If the step affected by inhibitor 2 precedes the step sensitive to inhibitor 1, or if these steps occur in parallel to each other, the requirement for the inhibitor 2–sensitive step must be fulfilled while the inhibitor 1–susceptible step is affected. In this case, the monitored event will proceed during the second stage. In contrast, if the step affected by inhibitor 2 occurs after the step sensitive to inhibitor 1, the monitored event will not proceed during the second stage, even in the presence of the compound that overcomes the block imposed by inhibitor 1. The same logic can be applied when the two inhibitors are added in the reverse order, namely when the exposure to inhibitor 2 precedes the treatment with inhibitor 1, which is supplemented together with the compound that overcomes the block imposed by inhibitor 2. Based on the results of the sequential treatment with two inhibitors added in two different orders, the decisive conclusion about the order of any pair of steps, which are sensitive to these inhibitors, can be made. In fact, if step one precedes step two, the monitored event will be affected only when the exposure to inhibitor 1 precedes the treatment with inhibitor 2. In contrast, if step one follows step two, the monitored hallmark event will be affected only when the exposure to inhibitor 1 follows the treatment with inhibitor 2.

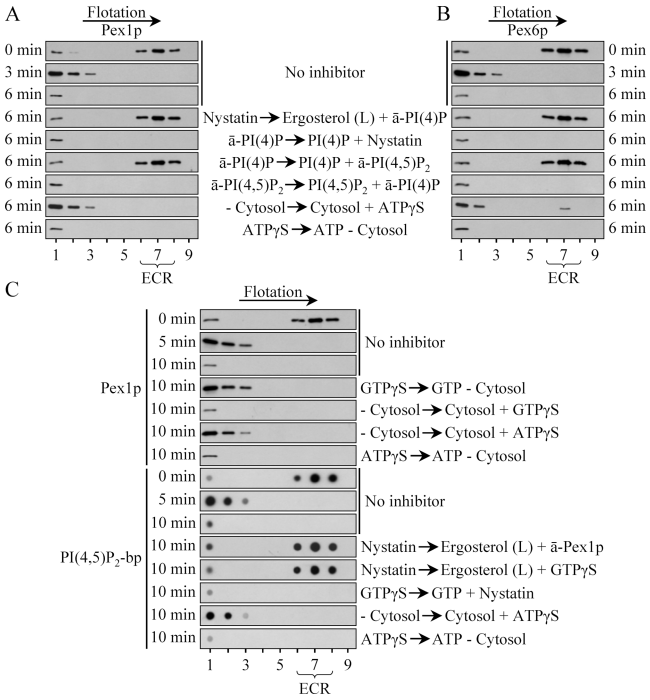


Figure S5. **The hierarchy of peroxisome priming- and docking-specific events that result in the relocation of Pex1p-, Pex6p-, and PI(4,5)P_2-binding proteins from ECR domains to a detergent-soluble portion of the membrane, followed by their release to the cytosol.** P1 (A) or P2 (B) vesicles were preincubated individually for 5 min at 26°C with or without nystatin (an ergosterol ligand), phosphoinositide-specific mAbs, or ATPγS in the presence or absence of cytosol, as indicated. Pretreated peroxisomal vesicles were then reisolated by centrifugation, washed, and resuspended in buffer containing liposomes with ergosterol, nystatin, phosphoinositides, phosphoinositide-specific antibodies, cytosol, ATPγS, or ATP, as indicated. After a 5-min incubation at 26°C, samples were supplemented with cytosol and ATP to yield standard fusion reactions and incubated at 26°C. Equal aliquots of peroxisomal vesicles were taken at the times indicated. P1 and P2 were pelleted by centrifugation. Samples were subjected to osmotic lysis, followed by centrifugation. The pellets of membranes recovered after such centrifugation were resuspended in ice-cold MBS buffer and supplemented with a detergent, Brij 35. After incubation on ice for 30 min, the Brij 35–treated membranes were subjected to centrifugation by flotation in a discontinuous sucrose density gradient. Proteins recovered in equal volumes of gradient fractions were resolved by SDS-PAGE and immunoblotted with antibodies to Pex1p (A) or Pex6p (B). (C) P1 and P2 were individually preprimed by incubation with cytosol and ATP for 10 min at 26°C. Peroxisomal vesicles were then reisolated by centrifugation, washed, and resuspended in a buffer. Reisolated P1 and P2 were incubated individually for 5 min at 26°C with or without GTPγS, ATPγS, or nystatin in the presence or absence of cytosol, as indicated. P1 and P2 were then reisolated by centrifugation, washed, and resuspended in buffer containing GTP, GTPγS, ATPγS, ATP, liposomes with ergosterol, antibodies to Pex1p, or nystatin in the presence or absence of cytosol, as indicated. After a 5-min incubation at 26°C, P1 and P2 were mixed and supplemented with cytosol and ATP to yield standard fusion reactions. Samples were incubated at 26°C. Equal aliquots of peroxisomal vesicles were taken at the times indicated. P1 and P2 were then pelleted by centrifugation. Samples were subjected to osmotic lysis, followed by centrifugation. The pellets of membranes recovered after such centrifugation were resuspended in ice-cold MBS buffer and supplemented with a detergent, Brij 35. After incubation on ice for 30 min, the Brij 35–treated membranes were subjected to centrifugation by flotation in a discontinuous sucrose density gradient. Proteins recovered in equal volumes of gradient fractions were resolved by SDS-PAGE and immunoblotted with antibodies to Pex1p. Equal volumes of gradient fractions were also subjected to protein-lipid overlay assays using nitrocellulose membrane arrays spotted with PI(4,5)P_2.

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