Dynamic ergosterol- and ceramide-rich domains in the peroxisomal membrane serve as an organizing platform for peroxisome fusion

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We describe unusual ergosterol- and ceramide-rich (ECR) domains in the membrane of yeast peroxisomes. Several key features of these detergent-resistant domains, including the nature of their sphingolipid constituent and its unusual distribution across the membrane bilayer, clearly distinguish them from well characterized detergent-insoluble lipid rafts in the plasma membrane. A distinct set of peroxisomal proteins, including two ATPases, Pex1p and Pex6p, as well as phosphoinositide- and GTP-binding proteins, transiently associates with the cytosolic face of ECR domains. All of these proteins are essential for the fusion of the immature peroxisomal vesicles P1 and P2, the earliest intermediates in a multistep pathway leading to the formation of mature, metabolically active peroxisomes. Peroxisome fusion depends on the lateral movement of Pex1p, Pex6p, and phosphatidylinositol-4,5-bisphosphate–binding proteins from ECR domains to a detergent-soluble portion of the membrane, followed by their release to the cytosol. Our data suggest a model for the multistep reorganization of the multicomponent peroxisome fusion machinery that transiently associates with ECR domains.

Introduction

Membrane fusion is vital for the division of eukaryotic cells, their communication with the environment via neurotransmitters and hormones, their integration into multicellular organisms, and their invasion by enveloped viruses (Jahn et al., 2003). Selective membrane fusion reactions inside eukaryotic cells maintain both the structural integrity of organelles during mitosis and the vesicular flow of proteins and lipids between organellar compartments of the secretory and endocytic pathways (Mayer, 2002). All membrane fusion reactions inside the secretory and endocytic systems of vesicular flow operate by very similar mechanisms and are served by a similar set of core protein components (Jahn et al., 2003). However, the fusion of mitochondria (Mozdy and Shaw, 2003) and peroxisomes (Titorenko and Rachubinski, 2001) do not require intracellular fusion machines that function in the secretory and endocytic pathways and may therefore involve unique, yet unknown, mechanisms (Mayer, 2002; Jahn et al., 2003).

We study membrane fusion with peroxisomes from the yeast *Yarrowia lipolytica*. In this yeast, peroxisome fusion is an initial step in a multistep pathway that leads to the formation of mature peroxisomes, P6, carrying the complete set of matrix and membrane proteins (Titorenko and Rachubinski, 2001). The pathway operates by conversion of five immature peroxisomal vesicles, termed P1 to P5, to mature peroxisomes in a temporally ordered manner from P1 to P6 (Guo et al., 2003). The immature peroxisomal vesicles P1 and P2, the earliest intermediates in the peroxisome assembly pathway, undergo fusion to generate larger vesicles, P3. Fusion between P1 and P2 in simple buffers containing ATP and supplemented with cytosol has been reconstituted in vitro (Titorenko et al., 2000). It is driven by ATP hydrolysis, requires cytosolic proteins, and depends on the peroxins Pex1p and Pex6p (Titorenko and Rachubinski, 2000), two AAA ATPases essential for peroxisome

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Abbreviations used in this paper: bp, binding proteins; ECR, ergosterol- and ceramide-rich; HLB, hydrophilic–lipophilic balance; n-OG, n-octyl-β-D-glucopyranoside; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine.

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biogenesis (Subramani et al., 2000; Purdue and Lazarow, 2001; Eckert and Erdmann, 2003).

Here, we investigate the effect of lateral heterogeneity of the peroxisomal membrane bilayer on the efficiency of the fusion between P1 and P2. We demonstrate that membrane bilayers of these peroxisomal vesicles exist in two lipid phases. A detergent-soluble phase is enriched in glycerophospholipids but contains only minor portions of ergosterol and ceramide. The other phase resists solubilization by various detergents. This phase is highly enriched in ergosterol and ceramide but has only traces of glycerophospholipid. We show that several key features of ergosterol- and ceramide-rich (ECR) domains in the peroxisomal membrane clearly distinguish them from lipid raft domains in the plasma membrane. ECR domains in the membranes of P1 and P2 are dynamic assemblies of a distinct set of lipids and proteins, including Pex1p, Pex6p, GTP-binding and hydrolyzing proteins, and proteins that specifically bind to certain phosphoinositides. Our findings provide a unique view of the multistep remodeling of the protein repertoire of ECR domains during fusion of P1 and P2. We define the hierarchy of individual steps during the spatial and temporal reorganization of the peroxisome fusion machinery that only transiently associates with ECR domains.

Results

Pharmacological analysis of peroxisome fusion

Using an in vitro assay for the fusion of the immature peroxisomal vesicles P1 and P2 (Titorenko et al., 2000), we identified several inhibitors of this process. Fusion of P1 and P2 was inhibited by nystatin, filipin III, and amphotericin B (Fig. 1 A), each known for its propensity to sequester sterol lipids (Simons and Toomre, 2000) but not fully remove them from the membrane (Foster et al., 2003). In the yeast *Saccharomyces cerevisiae* (Zinser et al., 1991) and *Y. lipolytica* (Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200409045/DC1), ergosterol is the major sterol lipid constituent of the peroxisomal membrane. The inhibitory effect of ergosterol ligands on peroxisome fusion can be reversed by phosphatidylcholine (PC)-based liposomes with ergosterol, but not by PC-based liposomes without it (Fig. 1 B). The inhibition of peroxisome fusion by ergosterol ligands was not due to the impairment of overall peroxisome integrity, as neither of these drugs caused the dissipation of the existing gradient of Ca^{2+} across the peroxisomal membrane (unpublished data). Together, these results suggest that ergosterol plays a specific role in the fusion of P1 and P2.

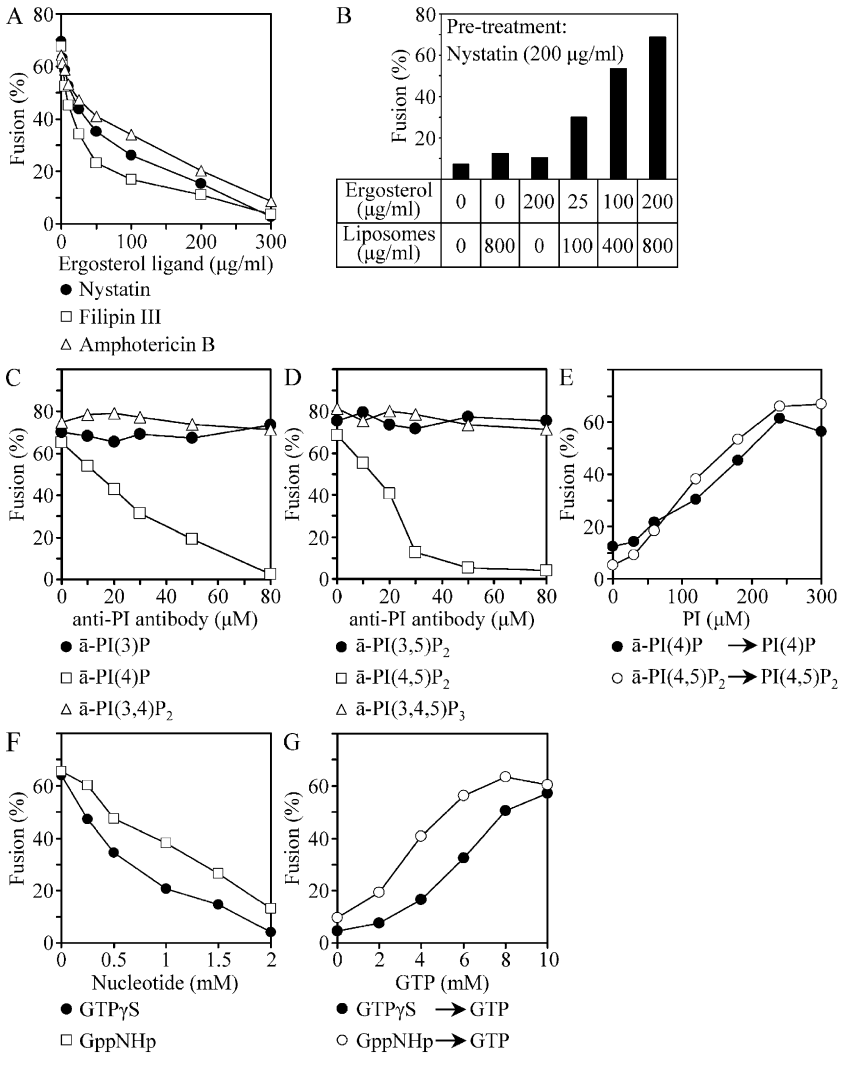

Figure 1. **Ergosterol ligands, phosphoinositide-specific antibodies, and nonhydrolyzable GTP analogues are reversible inhibitors of peroxisome fusion.** (A, C, D, and F) P1 and P2 were preincubated individually for 5 min at 26°C without inhibitor or with the indicated amounts of ergosterol ligands (A), phosphoinositide-specific antibodies (ā; C and D), or nonhydrolyzable GTP analogues (F). Pretreated P1 and P2 were mixed and supplemented with cytosol and ATP to yield standard fusion reactions. After a 90-min incubation at 26°C, the percentage of conversion of the precursor form of thiolase (pTHI) to its mature form (mTHI) as a measure of fusion was calculated. (B, E, and G) P1 and P2 were preincubated individually for 5 min at 26°C with 200 μg/ml nystatin (B), 60 μM of antibodies to PI(4)P or 60 μM of antibodies to $PI(4,5)P_2$ (E), or 2 mM GTPγS or 2 mM GppNHp (G). Pretreated P1 and P2 were then mixed. The samples were supplemented with cytosol and ATP to yield standard fusion reactions and incubated at 26°C in the presence or absence of liposomes without or with the indicated amounts of ergosterol (B), PI(4)P or $PI(4,5)P_2$ (E), or GTP (G). After a 90-min incubation, fusion efficiency was measured.

Monoclonal antibodies to the phosphoinositides phosphatidylinositol (PI)-4-phosphate (PI(4)P) and PI-4,5-bisphosphate ($PI(4,5)P_2$) inhibited peroxisome fusion (Fig. 1, C and D). The inhibition is reversible by externally added PI(4)P and $PI(4,5)P_2$, respectively (Fig. 1 E). In contrast, monoclonal antibodies to any other phosphoinositide tested did not affect peroxisome fusion (Fig. 1, C and D), showing the specificity of its inhibition by ligands of PI(4)P and $PI(4,5)P_2$. Therefore, only these two phosphoinositides are needed for the fusion of P1 and P2.

Two nonhydrolyzable GTP analogues, GTPγS and GppNHp, are reversible inhibitors of peroxisome fusion. Their negative effect on the fusion (Fig. 1 F) can be overturned by reisolating pretreated P1 and P2 and resuspending them in the standard fusion reaction mixture supplemented with GTP (Fig. 1 G). These data suggest that GTP hydrolysis by GTPase(s) is required for peroxisome fusion.

Dynamics of membrane-associated Pex1p, Pex6p, and phosphoinositide- and GTP-binding proteins (bp) during priming of P1 and P2

Fusion of P1 and P2 is a multistep process that includes priming, docking, and fusion events (Titorenko and Rachubinski, 2000). Priming (activation) of P1 and P2 before their physical contact commits both fusion partners to subsequent docking. Priming requires two AAA ATPases, Pex1p and Pex6p (Titorenko et al., 2000). Before priming, Pex1p is associated with the cytosolic surface of P1, whereas Pex1p and Pex6p are bound to the outer surface of P2. During priming, ATP hydrolysis triggers cytosol-dependent release of Pex1p from P1 and of Pex6p from P2, whereas P2-associated Pex1p remains bound to the organelle. We evaluated the requirements for the release of AAA ATPases during peroxisome priming. Using the extent of release of Pex1p from P1 and of Pex6p from P2 as a measure of priming efficiency, we found that, in addition to cytosolic proteins, ATP hydrolysis, and a particular type of AAA ATPase (Pex1p for P1 and Pex6p for P2; Titorenko and Rachubinski, 2000), priming of both fusion partners requires ergosterol, PI(4)P, and $PI(4,5)P_2$ (Fig. 2 A). However, priming does not depend on GTP hydrolysis (Fig. 2 A).

The observed susceptibility of peroxisome fusion to anti-PI(4)P and anti-$PI(4,5)P_2$ antibodies (Fig. 1, C and D) suggests that PI(4)P- and $PI(4,5)P_2$-bp associate with P1 and/or P2. Using PIP-Strips spotted with various phosphoinositides and phospholipids, we demonstrated that membrane proteins of P1 and P2 solubilized with a detergent, n-octyl-β-D-glucopyranoside (n-OG), bind only to PI(4)P and $PI(4,5)P_2$ but not to any other phosphoinositide or phospholipid tested (Fig. 2 B). PI(4)P- and $PI(4,5)P_2$-bp of intact peroxisomes were sensitive to digestion by external protease and were completely solubilized by 1 M NaCl (Fig. 2 B). Thus, these proteins are attached to the cytosolic surfaces of P1 and P2 through electrostatic interactions. Neither PI(4)P- nor $PI(4,5)P_2$-bp released from the outer faces of P1 and P2 during their priming (Fig. 2 B).

Peroxisome fusion was sensitive to nonhydrolyzable GTP analogues (Fig. 1 F), suggesting the involvement of P1-

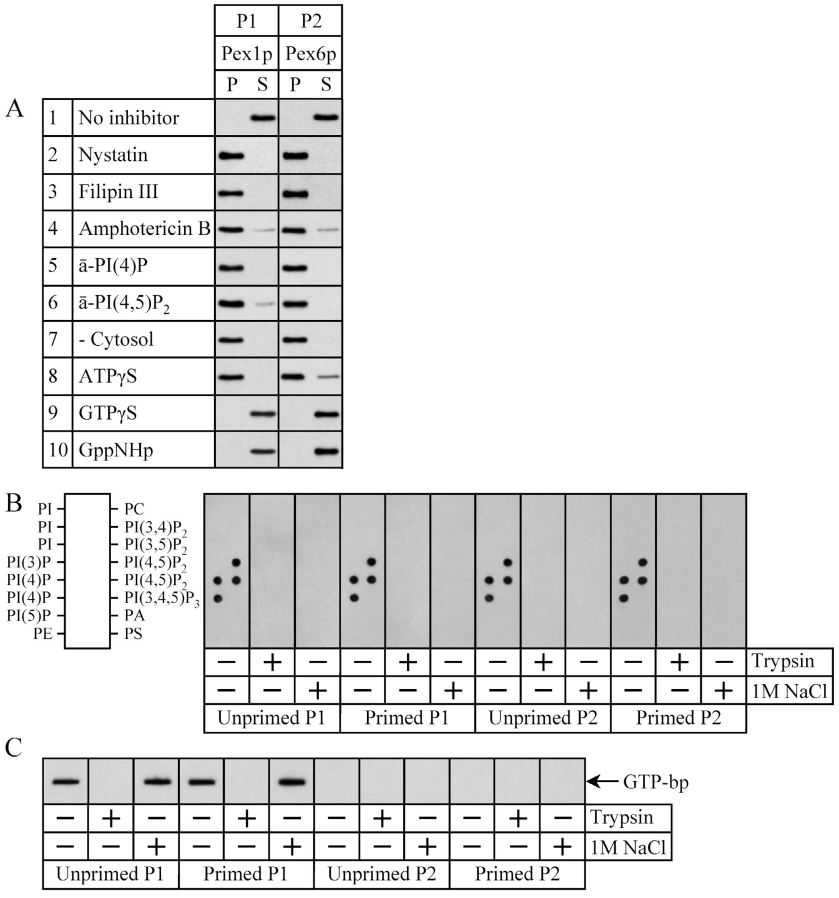

Figure 2. **Dynamics of the association of Pex1p, Pex6p, and phosphoinositide- and GTP-bp with membranes of P1 and P2 during their priming.** (A) *L*-[^{35}S]methionine–labeled P1 and *L*-[^{35}S]methionine–labeled P2 were preincubated individually for 5 min at 26°C with or without nystatin (an ergosterol ligand), phosphoinositide-specific antibodies, ATPγS, or nonhydrolyzable GTP analogues. Pretreated P1 and P2 were supplemented with ATP and incubated individually in the presence or absence of unlabeled cytosol, as indicated. After a 10-min incubation at 26°C, peroxisomal vesicles were pelleted. Pex1p and Pex6p were immunoprecipitated under denaturing conditions from the pellet (P) and supernatant (S) fractions. Immunoprecipitates were resolved by SDS-PAGE and visualized by fluorography. (B and C) Intact P1 and P2, either labeled with *L*-[^{35}S]methionine (B) or unlabeled (C), were primed individually by incubation with unlabeled cytosol and ATP or remained unprimed. Equal aliquots of primed and unprimed peroxisomes (30 μg of protein per aliquot) were treated with 30 μg of trypsin or 1 M NaCl for 30 min on ice. Peroxisomes were then osmotically lysed and subjected to centrifugation. The pellet of membranes recovered after such centrifugation was 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 (C). Lipid- and GTP-bp were visualized by autoradiography.

and/or P2-attached GTP-binding and hydrolyzing proteins in this process. The association of GTP-bp with P1 was confirmed by GTP slot-blot (Fig. 2 C). The observed susceptibility of the P1-attached GTP-bp to digestion by trypsin added to intact P1 and the inability of 1 M NaCl to solubilize the GTP-bp (Fig. 2 C) suggest that the association of GTP-bp with the outer face of P1 is not due to electrostatic interactions. We found that GTP-bp did not release from the outer face of P1 vesicles during their priming (Fig. 2 C).

Dynamics of membrane-associated Pex1p and phosphoinositide- and GTP-bp during docking of primed P1 and P2

The efficiency of peroxisome docking for fusion can be evaluated by monitoring the extent of in vitro formation of the docking complex P1/P2 (Titorenko et al., 2000). In this docking assay, P1 and P2 are first individually primed with cytosol and ATP, and then mixed and incubated with cytosol and ATP. Peroxisomes are finally subjected to fractionation by flotation on a multistep sucrose gradient. Under these conditions, the P1/P2 docking complex can be separated from undocked P1 and P2 and from P3, the product of fusion between P1 and P2 (Fig. 3; Titorenko et al., 2000). Using the in vitro assay for peroxisome docking, we found that, in addition to P2-bound Pex1p, cytosolic proteins, and ATP hydrolysis (Titorenko and Rachubinski, 2000), the docking of primed P1 and P2 depends on ergosterol, PI(4,5)P_2, and GTP hydrolysis by GTPase(s) (Fig. 3). However, docking does not require PI(4)P (Fig. 3).

The cytosol- and ATP hydrolysis-dependent release of P2-associated Pex1p from the membrane to the cytosol is a hallmark event of the docking of P1 and P2 (Titorenko and Rachubinski, 2000). We found that such release requires ergosterol and GTP hydrolysis but does not need PI(4)P or PI(4,5)P_2 (Fig. S1 A, available at http://www.jcb.org/cgi/content/full/jcb.200409045/DC1). We also examined the dynamics of association of PI(4)P- and PI(4,5)P_2-bp with P1 and P2 during their docking. PI(4,5)P_2-bp, but not PI(4)P-bp, dissociated from the outer faces of both fusion partners during docking (Fig. S1 B). The observed release of PI(4,5)P_2-bp from peroxisomes to the cytosol requires ergosterol, GTP hydrolysis by GTPase(s), cytosolic proteins, ATP hydrolysis, and Pex1p (Fig. S1 C). Finally, the GTP-bp remained associated with the outer face of P1 during docking (Fig. S1 D).

Requirements for the fusion of docked peroxisomal vesicles

To evaluate the requirements for the fusion of docked P1 and P2, the docking complex P1/P2 purified by flotation on a multistep sucrose gradient was incubated for 60 min with or without the inhibitors of the overall fusion process. Two nonhydrolyzable GTP analogues, GTPγS and GppNHp, impaired fusion between docked P1 and P2 (Fig. S1 E) but had no effect on the priming of fusion partners (Fig. 2). Of note, P1-associated GTP-bp remained bound to the organelle surface throughout the entire multistep fusion process (Fig. 2 and Fig. S1 D). These findings suggest a specific role for membrane-attached GTP-binding and hydrolyzing proteins in the fusion of docked P1 and P2. However, peroxisome fusion per se does not require ergosterol, PI(4)P, PI(4,5)P_2, cytosolic proteins, ATP hydrolysis, Pex1p, or Pex6p (Fig. S1 E), all of which are essential for the priming and/or docking of P1 and P2 (Figs. 2 and 3).

Differential solubility of Pex1p, Pex6p, and phosphoinositide- and GTP-bp in various detergents

Cholesterol in mammals (Simons and Toomre, 2000) and ergosterol in yeasts (Bagnat et al., 2000) are the major constituents of lipid rafts, which are dynamic domains of the plasma membrane that have been implicated in membrane protein trafficking, signal transduction, organization of the cytoskeleton, and pathogen internalization (Brown and London, 2000; Simons and Toomre, 2000; Mañes et al., 2003; Munro, 2003). Our observation that ergosterol ligands prevent the release of Pex1p and Pex6p from P1 and P2 vesicles (Fig. 2 A and Fig. S1), thereby inhibiting their priming and docking for fusion (Figs. 2 A and 3), suggests that ergosterol-rich domains, perhaps lipid raft(s), in the membranes of P1 and P2 carry both these AAA ATPases and somehow activate the peroxisome fusion machinery. Lipid rafts are defined operationally as membrane domains that are insoluble in cold nonionic detergents and are enriched in sterols, sphingolipids, and glycolipids (Brown and London, 2000). We evaluated the solubility of protein and lipid constituents of membranes of P1 and P2 in various detergents that differ in their hydrophilic–lipophilic balance (HLB). Membranes of unprimed P1 and P2 were extracted on ice with the nonionic detergent Brij 35, which has the highest HLB among the nonionic detergents tested (Fig. S2 C). After centrifugation of these detergent-treated membranes, a distinct set of Brij 35–insoluble proteins was sedimented (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200409045/DC1). The group of pelleted membrane proteins that resisted solubilization by Brij 35 includes the P1-associated forms of Pex1p, PI(4)P-bp, PI(4,5)P_2-bp, and GTP-bp (Fig. S2 A) and the P2-bound forms of Pex1p, Pex6p, PI(4)P-bp, and PI(4,5)P_2-bp (Fig. S2 B). In contrast, many peroxisomal membrane proteins, including the integral membrane protein Pex2p and the peripheral membrane protein Pex16p, were completely soluble in Brij 35 and in all other nonionic, ionic, and zwitterionic detergents tested (Figs. S2 and S3). Noteworthily, the ability of various nonionic detergents of the polyoxyethylene group to solubilize Pex1p and Pex6p is inversely proportional to their HLB values (Fig. S2 C). Together, these findings suggest that the observed propensity of Pex1p, Pex6p, and phosphoinositide- and GTP-bp to resist solubilization by Brij 35 was not due to an overall lower efficiency of Brij 35 as a detergent but reflected a specific phenomenon.

Of note, a nonionic detergent of the nonpolyoxyethylene group, n-OG, solubilized most of the membrane proteins, including Pex1p and Pex6p, that resisted solubilization by Brij 35 and other detergents (Figs. S2 and S3). Considering the demonstrated ability of n-OG to preserve the membrane-bound complexes of cytoskeletal proteins and their interacting protein partners (Röper et al., 2000), it is unlikely that the P1- and P2-bound membrane proteins associate with the cytoskeleton.

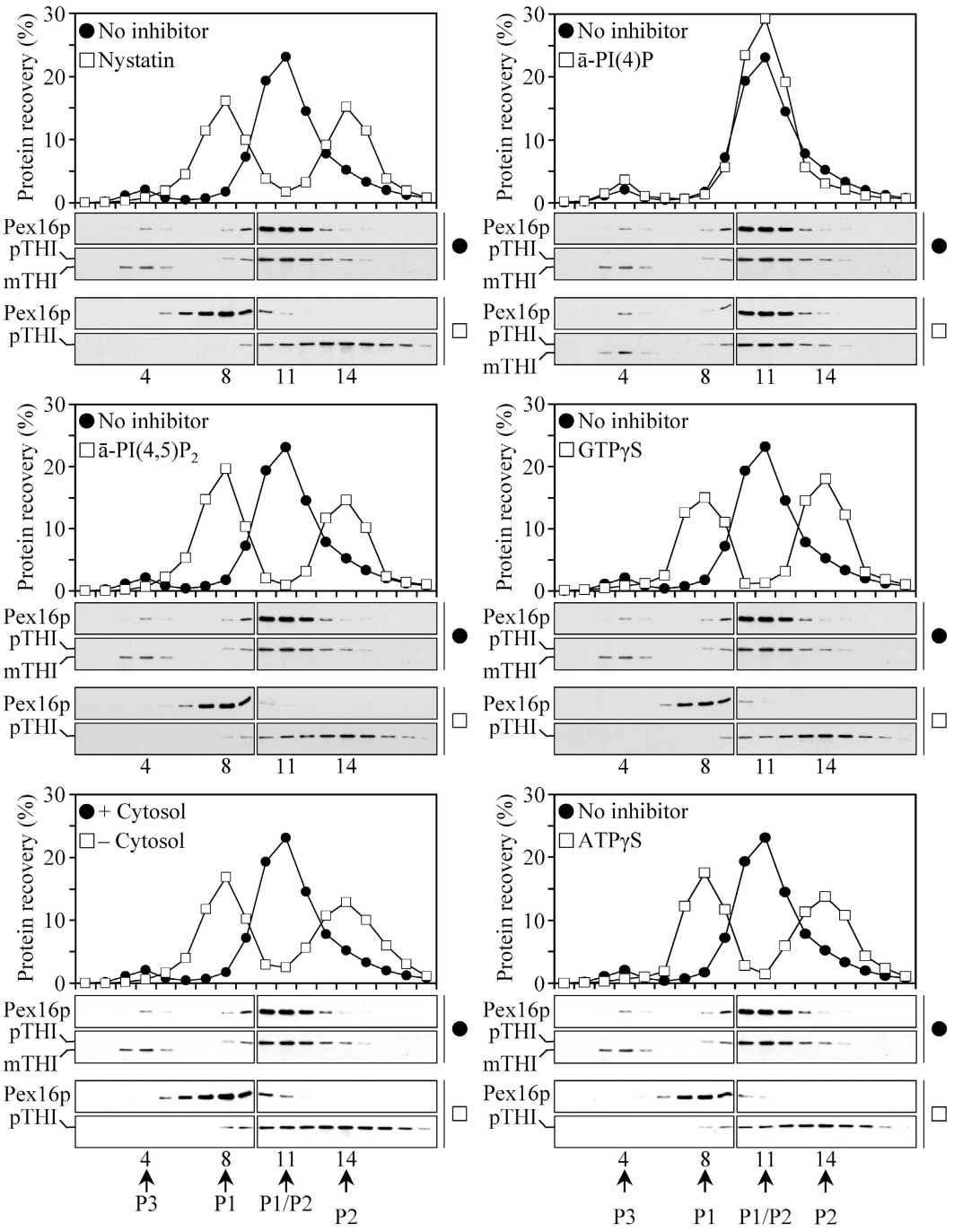

Figure 3. **Peroxisome docking requires ergosterol, PI(4,5)P_2, cytosolic proteins, and the hydrolysis of ATP and GTP.** P1 and P2 were individually preprimed by incubation with cytosol and ATP for 10 min at 26°C. Peroxisomal vesicles were reisolated by centrifugation, washed, and resuspended in buffer. Reisolated P1 and P2 were incubated individually for 5 min at 26°C with or without nystatin, phosphoinositide-specific mAbs, GTPγS, or ATPγS in the presence or absence of cytosol, as indicated. P1 and P2 were mixed and supplemented with ATP. After a 10-min incubation at 26°C, peroxisomal vesicles were subjected to fractionation by flotation on a multistep sucrose gradient. The percentage of recovery of loaded protein is presented. Equal volumes of gradient fractions were analyzed by immunoblotting with anti-Pex16p and anti-thiolase antibodies. The peak fractions for P1 and P2, their docking complex (P1/P2), and the product of their fusion (P3) are indicated. The positions of the precursor form of thiolase (pTHI), which was found in P2 and P1/P2, and of the mature form of thiolase (mTHI), which was associated with P3, are shown.

Thus, it seems that the observed insolubility of a distinct set of membrane proteins in Brij 35 was not due to their interactions with components of the cytoskeleton.

The insolubility of sterol- and sphingolipid-rich lipid rafts in cold nonionic detergents is due to the tight acyl chain packing and strong lipid–lipid interactions in these membrane domains (Brown and London, 2000). Ergosterol and the sphingolipid ceramide are the two major detergent-insoluble lipids in the membranes of P1 and P2 (Fig. S2). Only minute amounts of both these lipids were solubilized by all detergents tested, including Brij 35 (Fig. S2). In contrast, all five glycerophospholipids found in the membranes of P1 and P2, namely phosphati-

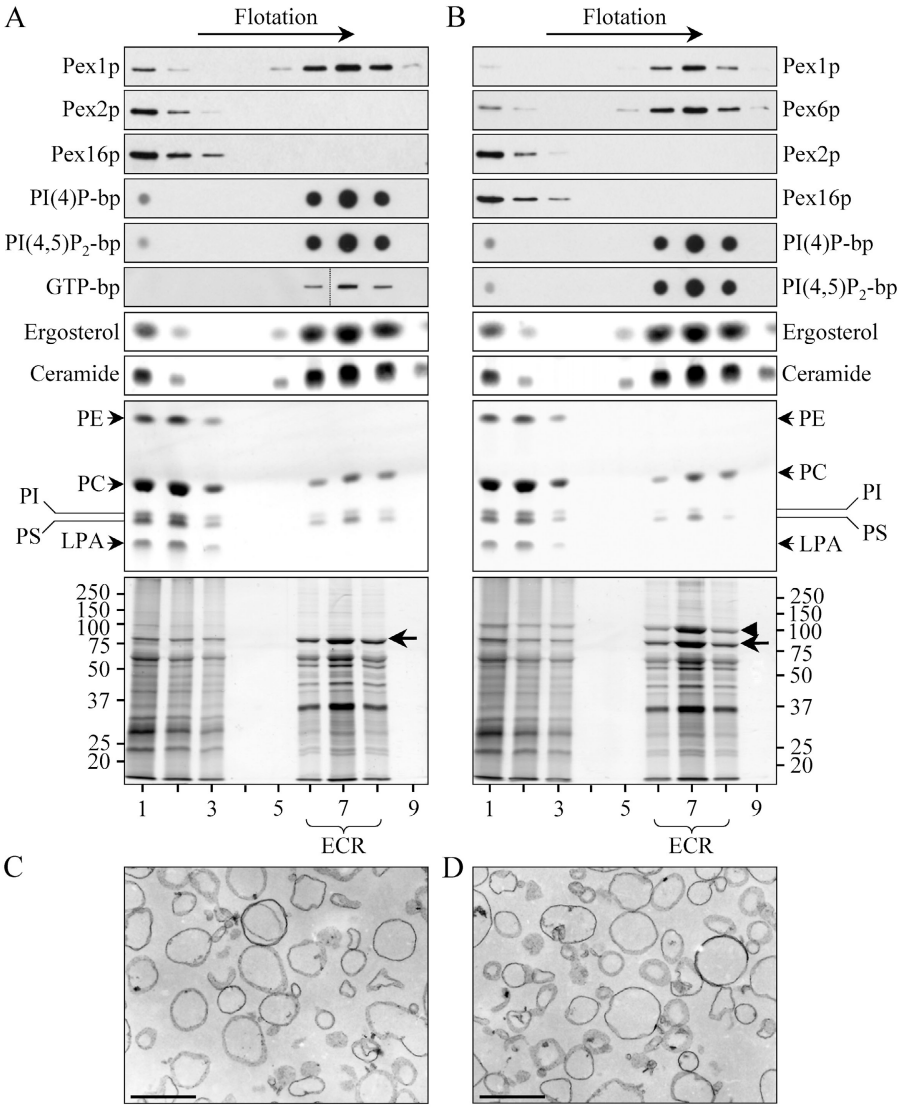

Figure 4. **Pex1p, Pex6p, and phosphoinositide- and GTP-bp associate with ECR membrane domains that can float to low density during centrifugation in a sucrose density gradient.** (A and B) The pellet of membranes recovered after centrifugation of osmotically lysed unprimed P1 (A) or P2 (B) was resuspended in ice-cold MBS buffer and supplemented with a nonionic 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 from equal volumes of gradient fractions were immunoblotted with the indicated antibodies. Equal volumes of gradient 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. The positions of Pex1p (arrows) and Pex6p (arrowhead), which were identified by mass spectrometric peptide mapping, are indicated. (C and D) Electron micrographs of ECR domains recovered in the low-density fraction 7 of the flotation sucrose density gradients presented in A and B, respectively. Bars, 100 nm.

dylethanolamine (PE), PC, PI, phosphatidylserine (PS), and lysophosphatidic acid, were mainly detergent-soluble, with only minor amounts of PC and PS resisting solubilization (Fig. S2).

Pex1p, Pex6p, and phosphoinositide- and GTP-bp reside in ECR membrane domains of unprimed P1 and P2

When exposed to cold nonionic detergents, detergent-insoluble protein and lipid components of lipid rafts can float to low density, away from detergent-soluble proteins and lipids, during centrifugation in sucrose density gradients (Brown and Rose, 1992). Our data on the insolubility of a distinct set of membrane proteins and lipids in various detergents (Figs. S2 and S3) suggested that these constituents of the membranes of P1 and P2 reside in ECR domains, perhaps lipid raft(s), that house several essential components of the peroxisome fusion machinery. To confirm the existence of such domains and to purify them for further characterization, Brij 35 extracts of the membranes of unprimed P1 and P2 were subjected to centrifugation by flotation in a discontinuous sucrose density gradient. A discrete group of detergent-insoluble membrane proteins, including the P1-associated forms of Pex1p, PI(4)P-bp, PI(4,5)P_2-bp, and GTP-bp (Fig. 4 A) and the P2-bound forms of Pex1p, Pex6p, PI(4)P-bp, and PI(4,5)P_2-bp (Fig. 4 B), floated to the low-density fractions 5–9 and peaked in fraction 7 of the gradient. The identity of Pex1p and Pex6p was confirmed by mass spectrometric peptide mapping. Furthermore, many detergent-soluble membrane proteins, including Pex2p and Pex16p, were recovered in the bottom fractions 1, 2, and 3 of the gradient (Fig. 4), with all three fractions corresponding to the load.

The bulk of the ergosterol and ceramide present in Brij 35 extracts of the membranes of unprimed P1 and P2 was recovered in the low-density fractions 5–9 of the flotation gradient (Fig. 4). In contrast, the major portion of each of the five glycerophospholipids associated with the membranes of P1 and P2 was soluble in the detergent and was found in the high-density bottom fractions 1–3 (Fig. 4). Only minor amounts of the glycerophospholipids PC and PS and traces of PI were seen in the low-density fractions 6–8 enriched in the components of

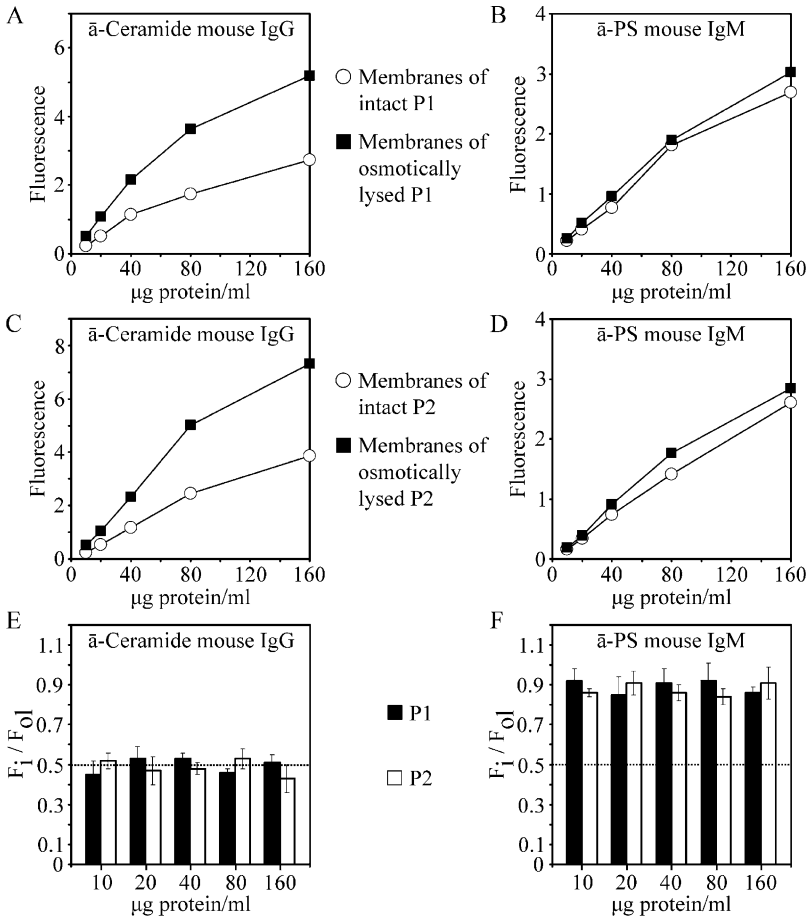

Figure 5. **Ceramide is distributed symmetrically between the two leaflets of the peroxisomal membrane, whereas phosphatidylserine (PS) associates mostly with the cytosolic leaflet.** (A–D) A suspension of purified P1 or P2 was divided into two equal aliquots. One aliquot remained untreated, whereas peroxisomal vesicles in the other aliquot were osmotically lysed. Twofold serial dilutions of intact P1 or P2 (from the first aliquot) and of the membranes recovered after centrifugation of osmotically lysed P1 or P2 (from the second aliquot) in the range of 10–160 μg of protein per milliliter were exposed to anti-ceramide mouse IgG or anti-PS mouse IgM. All samples were then treated with fluorescein-conjugated goat anti–mouse IgG or fluorescein-conjugated goat anti–mouse IgM antibodies. To amplify the signals from fluorescein-labeled secondary antibodies, the samples were first labeled with Alexa Fluor 488 rabbit anti-fluorescein/Oregon green IgG and then treated with Alexa Fluor 488 goat anti–rabbit IgG. The Alexa Fluor 488 fluorescence at 510 nm was monitored in individual samples. Controls were made for the nonspecific binding of mouse IgG, mouse IgM, and/or fluorescein- or Alexa Fluor 488–labeled antibodies to the membrane, and background fluorescence was subtracted. (E and F) The ratio "fluorescence for intact vesicles (F_i)/fluorescence for osmotically lysed vesicles (F_{ol})" (means ± SD from three experiments) was calculated for each dilution of intact P1 and P2 and of the membranes recovered after osmotic lysis of these peroxisomal vesicles. This ratio is equal to the fraction of the total pool of a monitored lipid that is located in the outer (cytosolic) leaflet of the membrane.

ECR domains, whereas PE and lysophosphatidic acid were not found in these fractions (Fig. 4). The Brij 35–insoluble structures purified from the membranes of P1 and P2 and recovered in the low-density fractions of the gradient appeared as 25–80-nm unilamellar vesicles of varied shape (Fig. 4, C and D).

Together, these results provide evidence for the existence of detergent-resistant ECR domains in the membranes of unprimed P1 and P2 vesicles. These domains (a) contain a distinct set of membrane proteins, including Pex1p, Pex6p, and several other essential components of the peroxisome fusion machinery; and (b) are highly enriched, as compared with a detergent-soluble portion of the peroxisomal membrane, in ergosterol and ceramide.

Lipid composition of the membranes of P1 and P2 and of their ECR domains

We examined the lipid makeup of the membranes of both fusion partners. Ergosterol and ceramide were at high levels in the membranes of unprimed P1 and P2. Ergosterol constituted 28–32 mol % of lipids in these membranes, whereas ceramide was present at 15–17 mol % (Fig. S4 A, available at http://www.jcb.org/cgi/content/full/jcb.200409045/DC1). ECR domains were substantially enriched in both these lipids as compared with the total membranes of P1 and P2. These detergent-resistant membrane domains contained 58–60 mol % of ergosterol and 29–31 mol % of ceramide (Fig. S4 B). In contrast, ECR domains were highly depleted in all five glycerophospholipids (Fig. S4 B). Accordingly, ergosterol/total glycerophospholipids and ceramide/total glycerophospholipids ratios for ECR domains greatly exceeded the ratios for the total membranes of P1 and P2 (Fig. S4 C). It seems that ECR domains represent a substantial fraction of the membranes of unprimed P1 and P2 vesicles, as 47–54 mol % of membrane lipids and 40–46% of membrane proteins were recovered in these domains (Fig. S4 D).

A sphingolipid component of ECR domains is distributed symmetrically between the two leaflets of the membrane

Lipids are asymmetrically arranged between the two leaflets of the plasma membrane bilayer in eukaryotic cells. Glycolipids and sphingomyelin, the two major sphingolipid components of lipid rafts in mammals, and the glycerophospholipid PC reside predominantly in the outer (exoplasmic) leaflet of the plasma membrane (Pomorski et al., 2004). In contrast, the glycerophospholipids PE, PI, and PS are restricted to the inner (cytosolic) leaflet of the plasma membrane (Pomorski et al., 2004). Cholesterol, a major sterol constituent of lipid rafts in mammals, is equally distributed across the bilayer (Munro, 2003). Using monoclonal antibodies to ceramide and PS, we evaluated the transbilayer distribution of these two lipids in the membranes of unprimed P1 and P2. In the membranes of osmotically lysed

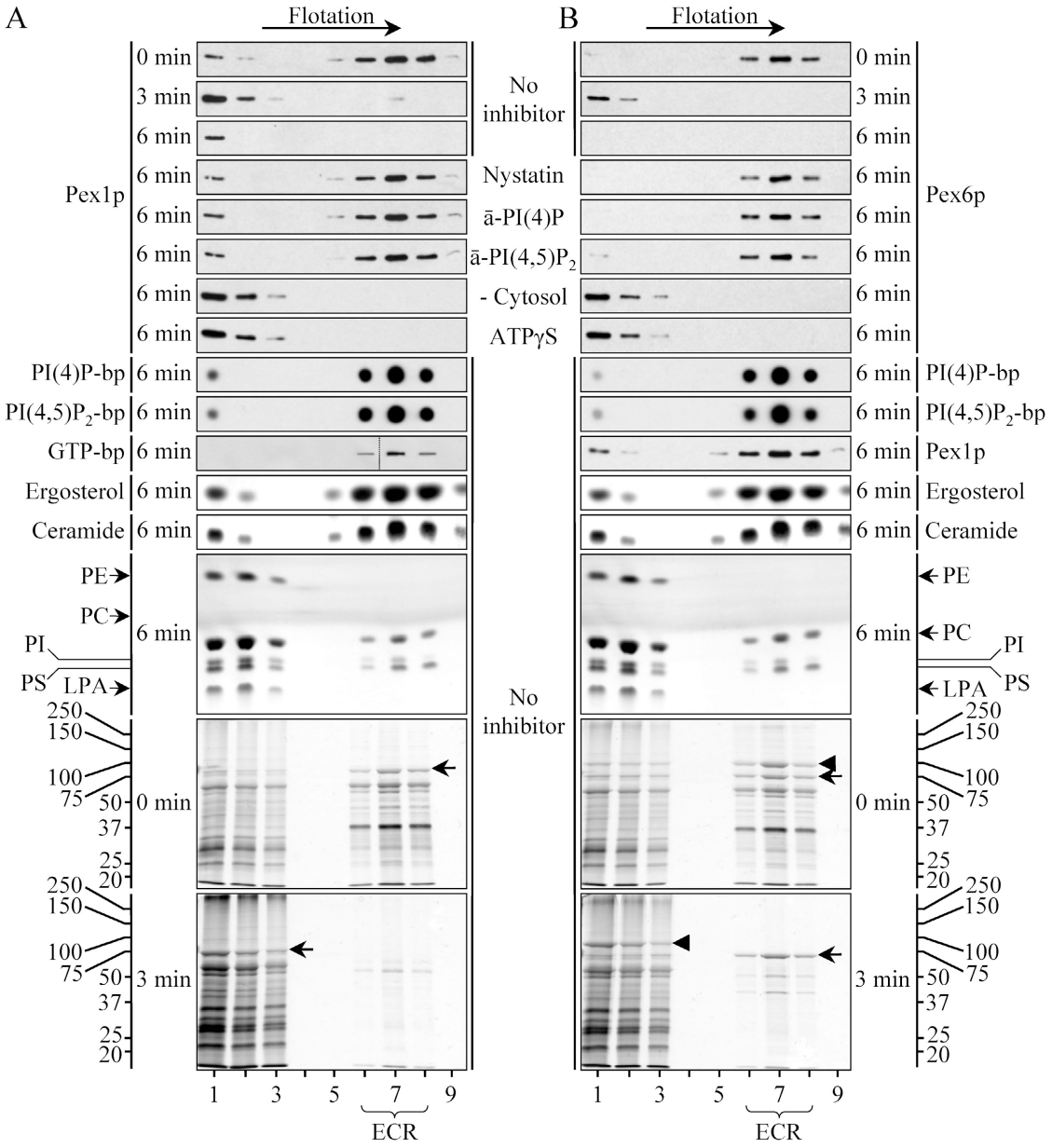

Figure 6. **Pex1p and Pex6p move from detergent-resistant ECR domains to a detergent-soluble portion of the membrane during peroxisome priming.** P1 (A) or P2 (B) vesicles were incubated individually with cytosol and ATP at 26°C. Equal aliquots of peroxisomal vesicles were taken at the times indicated. P1 and P2 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. Equal volumes of gradient fractions were analyzed as described in Fig. 4. The positions of Pex1p (arrows) and Pex6p (arrowheads), which were identified by mass spectrometric peptide mapping, are indicated.

P1 and P2, both leaflets of the bilayer were accessible to anti-ceramide and anti-PS antibodies. In contrast, in the membranes of intact P1 and P2, these monoclonal antibodies could detect only ceramide and PS that resided in the cytosolic leaflet. The levels of ceramide recovered in the membranes of osmotically lysed P1 and P2 exceeded the levels of this sphingolipid detected in intact membranes of both vesicles (Fig. 5, A and C), with about half of the ceramide located in the outer (cytosolic) leaflet of the bilayer (Fig. 5 E). Thus, the sphingolipid component of ECR domains is distributed symmetrically between the two leaflets of the membrane bilayers in P1 and P2. In contrast, the glycerophospholipid PS resides predominantly in the outer (cytosolic) leaflets of the membranes of P1 and P2. In fact, the vast majority of this lipid in intact P1 and P2 was accessible to anti-PS antibodies (Fig. 5, B, D, and F).

Peroxisome priming depends on the segregation of Pex1p and Pex6p from ECR domains

The release of P1-bound Pex1p and of P2-associated Pex6p from the organelle surface to the cytosol is a hallmark event of the priming of P1 and P2 (Titorenko and Rachubinski, 2000).

It seems that this event includes two consecutive steps. Specifically, both AAA ATPases initially relocate from ECR domains to an ergosterol- and ceramide-poor portion of the membrane, from which they are then released to the cytosol. In fact, when Brij 35 extracts of the membranes of unprimed P1 and P2 were subjected to centrifugation by flotation in a sucrose density gradient, the majority of P1-bound Pex1p and of P2-associated Pex6p was recovered in detergent-resistant ECR domains (Fig. 6). However, already by 3 min of priming, both proteins were seen only in detergent-soluble, ergosterol- and ceramide-poor membrane domains that were recovered in the high-density bottom fractions 1–3 of the gradient (Fig. 6). By 6 min of priming, both AAA ATPases were released from these detergent-soluble domains to the cytosol (Fig. 6). The two consecutive steps in the priming-specific release of Pex1p and Pex6p from the organelle surface to the cytosol have different requirements. Whereas the relocation of both proteins from ECR domains to an ergosterol- and ceramide-poor portion of the membrane depends on ergosterol, PI(4)P, and PI(4,5)P_2, their subsequent release to the cytosol requires cytosolic proteins and ATP hydrolysis (Fig. 6).

Priming of P1 and P2 led to dramatic changes in protein composition of ECR domains, with the majority of proteins being relocated from these domains to ergosterol- and ceramide-poor domains already by 3 min of priming (Fig. 6). Only a few proteins, including P1-bound GTP-bp, P2-associated Pex1p, and proteins that bind PI(4)P or PI(4,5)P_2 on the cytosolic faces of both fusion partners, remained associated with ECR domains (Fig. 6). However, the lipid repertoire of ECR domains did not undergo any noticeable remodeling during priming of P1 and P2 (compare Figs. 4 and 6).

To explore the hierarchy of individual steps during the priming-specific lateral movement of P1-bound Pex1p and of P2-associated Pex6p from ECR domains to ergosterol- and ceramide-poor domains, we developed a two-stage assay (see online supplemental Materials and methods). This assay examines whether or not the step affected by one inhibitor precedes, occurs in parallel, or follows the step sensitive to another inhibitor. In the two-stage assay for the priming-specific lateral movement of Pex1p and Pex6p in the membrane, the ergosterol-dependent step precedes the PI(4)P-requiring step. In fact, no such movement was seen when unprimed peroxisomes were initially exposed to nystatin, and then reisolated, washed, and exposed to anti-PI(4)P antibody in the presence of ergosterol-containing liposomes that overcome the block imposed by nystatin treatment (Fig. S5, A and B, available at http://www.jcb.org/cgi/content/full/jcb.200409045/DC1). In contrast, when nystatin and anti-PI(4)P antibody were added in the reverse order and PI(4)P was used during the second stage to overcome the block imposed by anti-PI(4)P antibody, the priming-specific lateral movement of Pex1p and Pex6p was not impaired and both AAA ATPases were successfully released to the cytosol (Fig. S5, A and B). Using the two-stage assay, we also established that the PI(4)P-dependent step during the priming-specific relocation of Pex1p and Pex6p to ergosterol- and ceramide-poor domains is followed by the PI(4,5)P_2-requiring step (Fig. S5, A and B).

The lateral movement of Pex1p and Pex6p to ergosterol- and ceramide-poor membrane domains during peroxisome priming is followed by their cytosol- and ATP hydrolysis-dependent release from these domains to the cytosol (Fig. 6). In the two-stage assay, such release of both AAA ATPases was impaired when unprimed peroxisomes were initially incubated in the presence of ATP, but in the absence of cytosol, and then reisolated and exposed to cytosol and the nonhydrolyzable analogue ATPγS (Fig. S5, A and B). In contrast, both AAA ATPases were successfully released to the cytosol when cytosolic proteins and ATPγS were added in the reverse order (Fig. S5, A and B). Thus, the cytosol-dependent step during this priming-specific event is a prerequisite for a step that needs ATP hydrolysis.

Segregation of PI(4,5)P_2-bp and of P2-bound Pex1p from ECR domains is mandatory for peroxisome docking

Docking of preprimed P1 and P2 results in further remodeling of the protein repertoire of their ECR domains. By 5 min of docking, P2-associated Pex1p and proteins that bind PI(4,5)P_2 on the cytosolic faces of both fusion partners moved from these floating membrane domains to detergent-soluble, ergosterol- and ceramide-poor domains recovered in the high-density bottom fractions of the flotation gradient (Fig. 7). The movement of Pex1p and PI(4,5)P_2-bp to a detergent-soluble portion of the membranes was followed by the release of these proteins to the cytosol, which was evident after 10 min of docking (Fig. 7). Not all proteins moved away from ECR domains during peroxisome docking. The group of ECR resident proteins included P1-associated GTP-bp and proteins that bind PI(4)P on the cytosolic faces of both fusion partners (Fig. 7). Furthermore, no dramatic changes in lipid composition of ECR domains were observed during docking of separately primed P1 and P2 (compare Figs. 6 and 7).

Whereas the segregation of P2-associated Pex1p from ECR domains was prevented by nystatin treatment and, therefore, requires ergosterol, the subsequent release of this AAA ATPase to the cytosol depends on cytosolic proteins and on the hydrolysis of both ATP and GTP (Fig. 7). In the two-stage assay for the docking-specific release of Pex1p to the cytosol, the GTP hydrolysis-dependent step precedes a step that requires cytosolic proteins. In fact, Pex1p was not released to the cytosol when separately primed P1 and P2 were mixed, treated with the nonhydrolyzable analogue GTPγS in the presence of cytosol, and reisolated and exposed to GTP, which overcomes the block imposed by GTPγS treatment (Fig. S5 C). In contrast, the release of Pex1p to the cytosol was not compromised when separately primed P1 and P2 were mixed, incubated in the absence of cytosol, and reisolated and exposed to cytosol and GTPγS (Fig. S5 C). Using the two-stage assay, we also established that the cytosol-dependent step during the docking-specific release of Pex1p to the cytosol is followed by a step that requires ATP hydrolysis (Fig. S5 C).

Another docking-specific event, the lateral segregation of PI(4,5)P_2-bp from ECR domains, requires ergosterol, Pex1p, and GTP hydrolysis (Fig. 7). In the two-stage assay,

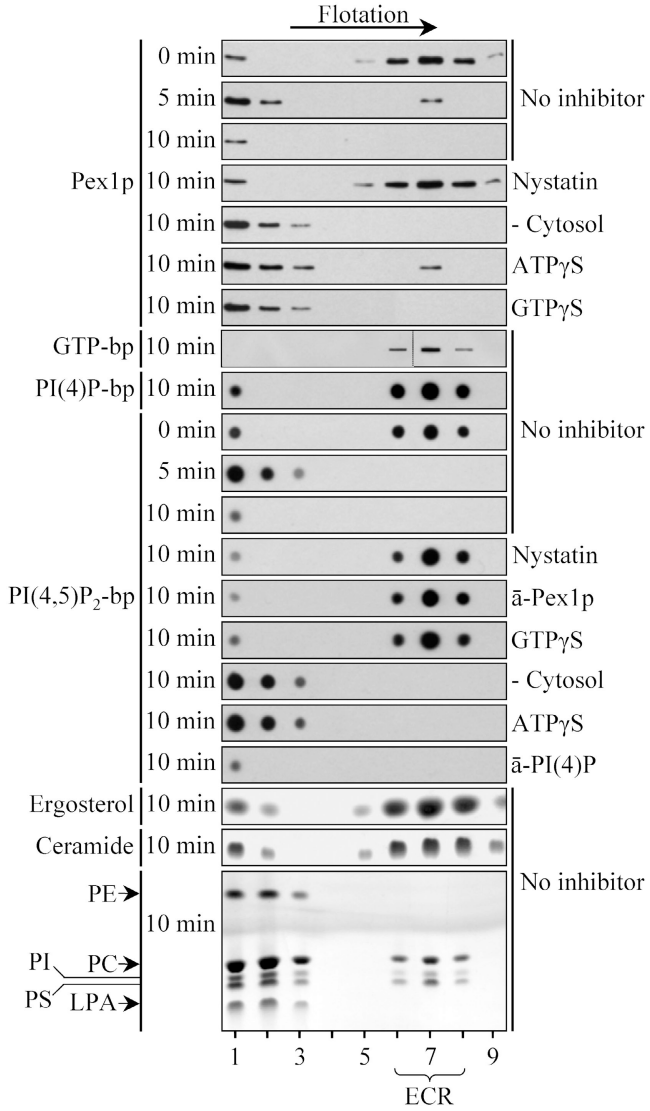

Figure 7. **PI(4,5)P_2-bp and P2-bound Pex1p segregate from ECR domains during peroxisome docking.** P1 and P2 were incubated individually with cytosol and ATP at 26°C. After a 10-min incubation, P1 and P2 were pelleted, resuspended in a buffer, and mixed. Samples were incubated at 26°C with or without cytosol, ATP, nystatin, GTPγS, ATPγS, antibodies to Pex1p, or antibodies to PI(4)P. Equal aliquots of peroxisomal vesicles were taken at the times indicated. 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. Equal volumes of gradient fractions were analyzed as described in Fig. 4.

both the Pex1p- and the GTP hydrolysis-dependent steps follow a step that requires ergosterol. Indeed, the PI(4,5)P_2-bp did not move to a detergent-soluble portion of the membrane when the exposure of separately primed and mixed P1 and P2 to nystatin preceded the treatment with monospecific antibodies to Pex1p or with GTPγS added together with ergosterol-containing liposomes to overcome the block imposed by nystatin exposure (Fig. S5 C). Noteworthily, in the beginning of peroxisome docking, PI(4,5)P_2-bp are attached to both fusion partners (Fig. 2), Pex1p can only be found on the cytosolic face of P2 (Fig. 6), and the only GTP-bp that can be detected is the one that resides on the outer surface of P1 (Fig. 2). Together, these findings suggest that the docking-specific segregation of P1-bound PI(4,5)P_2-bp from ECR domains requires GTP-bp, whereas such segregation of P2-associated PI(4,5)P_2-bp depends on Pex1p.

The last stage in the process of spatial rearrangement of PI(4,5)P_2-bp on the cytosolic surfaces of both fusion partners involves their cytosol- and ATP hydrolysis-dependent release from a detergent-soluble portion of the membrane to the cytosol (Fig. 7). Using the two-stage assay, we found that the cytosol-dependent step during such release of PI(4,5)P_2-bp is followed by a step that requires ATP hydrolysis (Fig. S5 C).

Discussion

Dynamics of the peroxisome fusion machinery

The results of this work and our published data (Titorenko and Rachubinski, 2000) suggest the following model for the multistep remodeling of the peroxisome fusion machinery in the membranes of P1 and P2 (Fig. 8). In unprimed P1 and P2, all identified essential components of this machinery, including Pex1p, Pex6p, PI(4)P-bp, PI(4,5)P_2-bp, and GTP-bp, are attached to the cytosolic surface of ECR domains. Priming of fusion partners is initiated by the lateral movement of P1-associated Pex1p and P2-bound Pex6p from ECR domains to an ergosterol- and ceramide-poor portion of the membrane. This essential event in the process of activating fusion partners for subsequent docking includes at least three consecutive steps. The initial ergosterol-dependent step is followed by a PI(4)P-dependent step, which precedes a step that requires PI(4,5)P_2. After their segregation from ECR domains, both P1-associated Pex1p and P2-bound Pex6p are released to the cytosol. Such release of both AAA ATPases from an ergosterol- and ceramide-poor portion of the membrane is mandatory for the priming of both fusion partners and includes two steps. The first step requires cytosolic proteins, whereas the next step is driven by ATP hydrolysis. Priming of P1 and P2 is followed by their docking. Docking of primed peroxisomal vesicles is a multistep process, which begins with the lateral movement of PI(4,5)P_2-bp in the membranes of P1 and P2 from ECR domains to ergosterol- and ceramide-poor domains. This lateral movement of PI(4,5)P_2-bp occurs in three consecutive steps. The first step needs ergosterol in the membranes of both fusion partners. The second step depends on Pex1p that resides in ECR domains of P2 vesicles. The third step requires GTP hydrolysis by GTPase(s), perhaps by GTP-bp in ECR domains of P1 vesicles. The docking-specific lateral movement of PI(4,5)P_2-bp in the membranes of P1 and P2 is followed by the ergosterol-dependent relocation of P2-bound Pex1p from ECR domains to ergosterol- and ceramide-poor domains. After their lateral movement to ergosterol- and ceramide-poor portions of the membranes of both fusion partners, P1-associated PI(4,5)P_2-bp and P2-bound PI(4,5)P_2-bp and Pex1p are released to the cytosol. Such release of PI(4,5)P_2-bp and Pex1p begins with a cytosol-dependent step, which is fol-

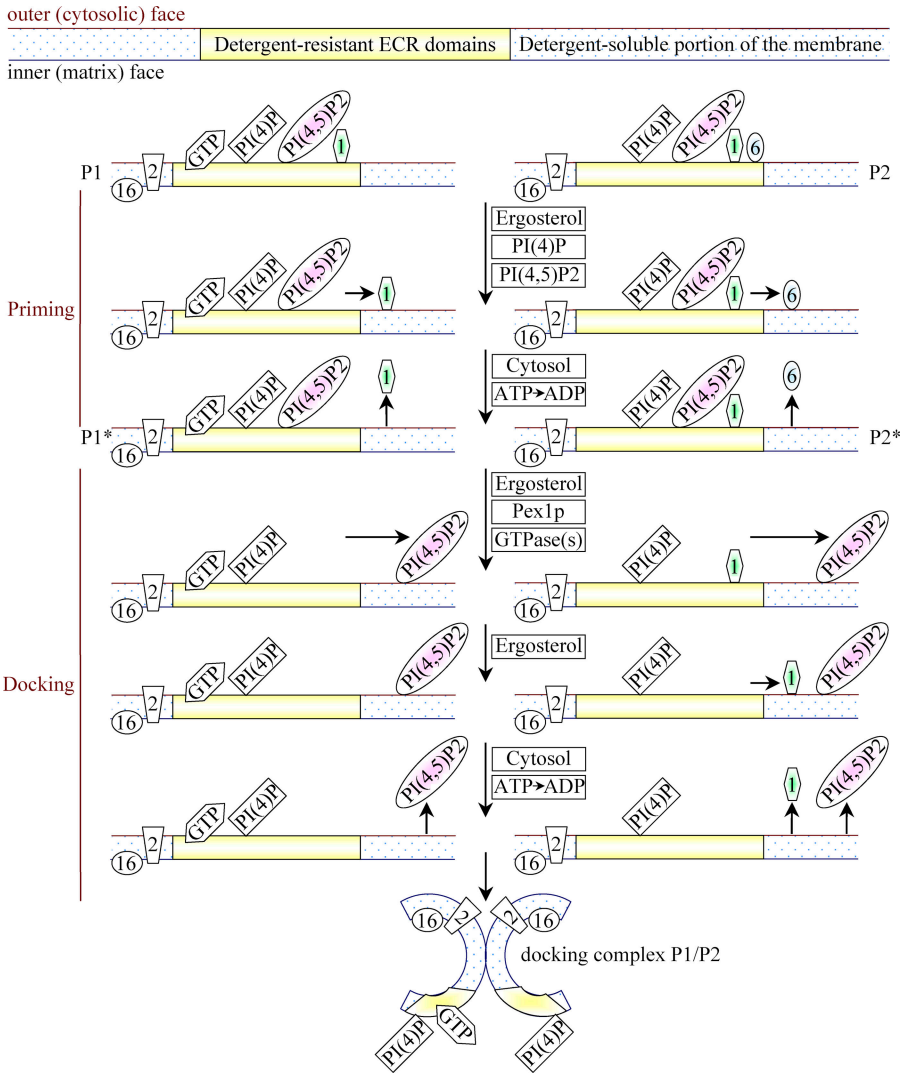

Figure 8. **A model for the multistep remodeling of the peroxisome fusion machinery.** The multicomponent peroxisome fusion machinery, which only transiently resides in ECR membrane domains of P1 and P2 vesicles, undergoes multiple rounds of temporal and spatial reorganization during priming and docking of both fusion partners. See Discussion for details.

lowed by a step that requires ATP hydrolysis. By the end of the docking process, PI(4)P-bp and GTP-bp remain in ECR domains of P1 and P2. The GTP-bp, together with calmodulin and a protein machinery that maintains the Ca^{2+} gradient across the peroxisomal membrane, is mandatory for the fusion of docked fusion partners (unpublished data). It remains to be established how the described remodeling of the peroxisome fusion machineries in the membranes of P1 and P2 changes the physical properties and topology of lipid bilayers in which these machineries operate, thereby triggering peroxisome docking. The exact nature of the changes in the membrane lipid bilayers of both fusion partners is currently being investigated.

Do ECR domains exist in the peroxisomal membrane or are they an artifact of the detergent insolubility assay?

The assay for insolubility of protein and lipid constituents of cellular membranes in cold detergents has been widely used for the identification of lipid rafts (Brown and Rose, 1992; Foster et al., 2003). The validity of this assay has been demonstrated by the identification of numerous detergent-resistant membrane assemblies of proteins and lipids that have been implicated in a variety of essential cellular processes (Simons and Toomre, 2000; Mañes et al., 2003). However, recent findings suggested potential caveats associated with the interpretation of results of the detergent insolubility assay (Munro, 2003). It was concluded that only a decrease or an increase in the detergent insolubility of a membrane protein at different consecutive steps of a cellular process could provide proof for the lateral movement of the protein of interest from one membrane domain to another (Munro, 2003; Mayor and Rao, 2004). We used the detergent solubilization approach to study the dynamics of temporal and spatial reorganization of the multicomponent peroxisome fusion machinery in the membranes of P1 and P2. We found that a distinct set of protein components of this machinery in unprimed P1 and P2 resisted solubilization by the detergent Brij 35. Individual components of the fusion machinery became detergent-soluble, and were eventually released to the cytosol, at different steps of the peroxisome fusion process. Moreover, we defined a hierarchy in which various inhibitors of peroxisome fusion selectively affected the lateral movement

of their protein targets in the peroxisomal membrane. Together, these findings provide evidence that detergent-resistant ECR domains in the membranes of P1 and P2 do not represent an artifact of the detergent insolubility assay. On the contrary, these membrane domains exist as dynamic assemblies of a distinct set of proteins and lipids whose remodeling during peroxisome priming and docking is mandatory for the fusion of peroxisomal membranes.

Unique properties of ECR domains in the peroxisomal membrane

Some properties of ECR domains in the membranes of P1 and P2 vesicles distinguish them from well characterized lipid raft domains in the plasma membrane. Sphingolipids of lipid rafts in the plasma membrane have large polar head groups that are attached to their sphingosine base (Sprong et al., 2001). In contrast, no polar head group is attached to the sphingosine base of ceramide (Sprong et al., 2001), an abundant sphingolipid component of ECR domains. It should be noted that ceramide in model membranes forms detergent-insoluble lipid domains that are significantly more stable than those formed in the presence of plasma membrane sphingolipids (Xu et al., 2001). Moreover, by stabilizing lipid raft domains in ER membranes, ceramide could enhance the association of glycosylphosphatidylinositol-anchored proteins with lipid rafts, thereby promoting selective sorting of these proteins into vesicles distinct from those carrying many other secretory and plasma membrane proteins (Mayor and Riezman, 2004). Whether or not ceramide could promote the assembly of the ECR domain-based peroxisome fusion machinery in unprimed P1 and P2 remains to be elucidated.

Another distinct feature of ECR domains is the unusual distribution of their sphingolipid component, ceramide, across the membrane bilayers in P1 and P2. In the plasma membrane, sphingolipids are restricted to the outer leaflet (Pomorski et al., 2004), as they are unable to move across the bilayer (Sprong et al., 2001). These lipids cluster with cholesterol, which preferentially interacts with sphingolipids rather than glycerophospholipids, thereby forming distinct lipid raft domains in the outer leaflet of the plasma membrane (Munro, 2003). In contrast, in the membranes of P1 and P2, the sphingolipid ceramide is distributed symmetrically between the two leaflets of the bilayers. The bulk of ceramide, which spontaneously flips across the membrane bilayer with a half-time of $\sim$10 min (Sprong et al., 2001), is in ECR domains of the membranes of P1 and P2. It remains to be seen if the symmetric distribution of ceramide across the peroxisomal membrane and its ability to flip between the two leaflets of the bilayer promote the coordination of events that occur in the cytosolic and lumenal leaflets of ECR domains.

ECR domains in the peroxisomal membrane and lipid raft domains in the plasma membrane have two important features in common. First, ECR domains constitute a significant portion of the membranes of unprimed P1 and P2, with about half of membrane lipids and proteins being recovered in these membrane domains. Lipid rafts in the plasma membrane also represent a substantial fraction of the membrane (Pierini and Maxfield, 2001). In certain cells, the plasma membrane resembles a dense assembly of numerous types of small lipid rafts that, once cells are stimulated, form larger assemblies (or flotillas; Pierini and Maxfield, 2001). Whether or not ECR domains in the peroxisomal membrane represent several distinct types of ECR microdomains, which differ in their protein composition and collide in response to certain stimuli, remains to be elucidated. Second, both ECR domains in the peroxisomal membrane and lipid rafts in the plasma membrane are dynamic. When P1 and P2 vesicles are stimulated for priming and docking, numerous protein constituents of ECR domains rapidly move from these domains to an ergosterol- and ceramide-poor portion of the membrane. Likewise, lipid raft proteins in the plasma membrane are extremely mobile and undergo rapid lateral diffusion even in unstimulated cell membranes (Kenworthy et al., 2004).

The domain organization of intracellular membranes is vital for many cellular processes

Lipid raft domains in mammals, which are formed in the lumenal leaflet of the Golgi membrane (Sprong et al., 2001), have been implicated in the selective protein sorting to the apical surface of polarized epithelial cells (Slimane et al., 2003), the retention of Golgi-resident proteins (Munro, 2003), and the formation and maintenance of the Golgi cisterna (Helms and Zurzolo, 2004). Lipid raft domains in the yeast *S. cerevisiae*, which are formed in the ER (Bagnat et al., 2000), may function in the ER-to-Golgi vesicular transport of glycosylphosphatidylinositol-anchored proteins (Mayor and Riezman, 2004). Furthermore, the clustering of certain components of the membrane fusion apparatus in cholesterol-enriched membrane domains is essential for exocytosis, the process by which secretory vesicles fuse with the plasma membrane (Salaün et al., 2004). Our findings provide evidence that, similarly to intracellular compartments of secretory/endocytic pathways, peroxisomes in the yeast *Y. lipolytica* contain sterol- and sphingolipid-rich membrane domains. These ECR domains orchestrate a particular cellular process: the fusion of peroxisomal vesicles P1 and P2. Together, these data strongly suggest that the segregation of certain proteins and lipids into distinct domains in intracellular membranes is essential for the biogenesis of eukaryotic organelles.

In conclusion, we have identified unusual ECR domains in the membranes of the immature peroxisomal vesicles P1 and P2. These ECR membrane domains exist as dynamic assemblies of a distinct set of proteins and lipids that resist solubilization by cold detergents. ECR domains function as an organizing platform for the fusion of P1 and P2. We suggest a model for the dynamics of temporal and spatial reorganization of the protein team that transiently resides in ECR domains and controls peroxisome fusion. The mechanisms by which individual protein and lipid components of ECR domains regulate the stepwise remodeling of the peroxisome fusion machinery are currently being investigated.

Materials and methods

The *Y. lipolytica* wild-type strain *P01d* (Guo et al., 2003), the media and growth conditions for *Y. lipolytica* (Titorenko et al., 1998), and antibodies to Pex1p, Pex2p, Pex6p, Pex16p, and thiolase (Titorenko et al., 2000) have been previously described. Purification of the immature peroxisomal vesicles P1 and P2 (Titorenko et al., 2000), radiolabeling of cells (Titorenko et al., 1998), and GTP slot-blot (Wagner et al., 1992) were performed as described previously. To isolate ECR domains, 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. The suspension of membranes was then supplemented with a detergent, Brij 35. 5 mg of detergent was used for the treatment of 1 mg of peroxisomal membrane protein. After incubation on ice for 30 min, a 300-μl sample of Brij 35–treated membranes was transferred to the bottom of a 5-ml ultraclear centrifuge tube (Beckman Coulter) and supplemented with four volumes of 65% (wt/wt) sucrose in ice-cold MBS buffer to adjust the sucrose concentration of the sample to 52% (wt/wt). The sample was overlaid with 600 μl of 30% sucrose, 600 μl of 25% sucrose, 600 μl of 20% sucrose, 600 μl of 15% sucrose, 600 μl of 10% sucrose, and 500 μl of 5% sucrose (all wt/wt in MBS buffer). After centrifugation at 200,000 *g* for 18 h at 4°C in a rotor (model SW50.1; 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 or silver staining. Equal volumes of gradient fractions were also subjected to the protein-lipid overlay assay, GTP slot-blot, lipid extraction, and TLC.

Online supplemental material

The online version of this manuscript contains supplemental Materials and methods and additional figures (Figs. S1–S5). Supplemental Materials and methods describe reagents, preparation of ergosterol-containing liposomes, detergent treatment of peroxisomal membranes, protein-lipid overlay assays, lipid analyses, mass spectrometry, and a two-stage assay for defining the hierarchy of membrane-associated events during peroxisome priming and docking. Fig. S1 provides data on the dynamics of the association of Pex1p and phosphoinositide- and GTP-bp with membranes of primed P1 and P2 during their docking and outlines the requirements for the fusion of docked P1 and P2. Fig. S2 summarizes data on the effect of various detergents on the solubility of proteins and lipids associated with the membranes of unprimed P1 and P2. Fig. S3 shows the spectra of detergent-soluble and -insoluble membrane proteins that associate with P1 and P2. Fig. S4 provides data on the lipid composition of the membranes of unprimed P1 and P2 and of their ECR domains. Fig. S5 summarizes data on the hierarchy of peroxisome priming- and docking-specific events that result in the segregation of Pex1p, Pex6p, and PI(4,5)P_2-bp from ECR domains, followed by their release to the cytosol. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200409045/DC1.

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