A Four-Step Cycle Driven by PI(4)P Hydrolysis Directs Sterol/PI(4)P Exchange by the ER-Golgi Tether OSBP

Bruno Mesmin,1,3 Joëlle Bigay,1,3 Joachim Moser von Filseck,1 Sandra Lasca Gervais,2 Guillaume Drin,1 and Bruno Antonny1,*

1Institut de Pharmacologie Moléculaire et Cellulaire, Université Nice Sophia Antipolis and CNRS, 06560 Valbonne, France
2Centre Commun de Microscopie Appliquée, Université Nice Sophia Antipolis, Parc Valrose, 06900 Nice, France
3These authors contributed equally to this work

*Correspondence: antonny@ipmc.cnrs.fr
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SUMMARY

Several proteins at endoplasmic reticulum (ER)-Golgi membrane contact sites contain a PH domain that interacts with the Golgi phosphoinositide PI(4)P, a FFAT motif that interacts with the ER protein VAP-A, and a lipid transfer domain. This architecture suggests the ability to both tether organelles and transport lipids between them. We show that in oxysterol binding protein (OSBP) these two activities are coupled by a four-step cycle. Membrane tethering by the PH domain and the FFAT motif enables sterol transfer by the lipid transfer domain (ORD), followed by back transfer of PI(4)P by the ORD. Finally, PI(4)P is hydrolyzed in cis by the ER protein Sac1. The energy provided by PI(4)P hydrolysis drives sterol transfer and allows negative feedback when PI(4)P becomes limiting. Other lipid transfer proteins are tethered by the same mechanism. Thus, OSBP-mediated back transfer of PI(4)P might coordinate the transfer of other lipid species at the ER-Golgi interface.

INTRODUCTION

Membrane contact sites (MCSs) are regions where the membranes of two organelles are closely apposed, typically 10–20 nm apart (Friedman and Voeltz, 2011; Helle et al., 2013; Levine, 2004). In a few cases, such structures are obvious by thin-section electron microscopy (EM) as the two membranes are aligned along significant distances. The best examples are contact sites between the endoplasmic reticulum (ER) and the plasma membrane (PM) in yeast (Manford et al., 2012; West et al., 2011). In most cases, however, more elaborate morphological approaches are required; for example, EM tomography of cryo-fixed preparations identified MCSs between a specialized region of the ER, called trans ER, and the trans Golgi (Ladinsky et al., 1999).

MCSs are diverse, but a recurrent observation is the involvement of the ER (Friedman and Voeltz, 2011; Helle et al., 2013; Levine, 2004). By making an extensive network, the ER is indeed in the best position to contact other cellular organelles. In addition, because the ER is the main site for lipid synthesis, MCSs suggest ways to supply lipids to a second membrane with limited lipid synthesis ability. This hypothesis is supported by the presence of proteins with lipid exchange activity in many MCSs (Helle et al., 2013; Lev, 2010; Levine, 2004).

MCSs between the ER and the Golgi contain the following lipid transporters: CERT, which transports ceramide (Hanada et al., 2003); FAPP2, which transports glucosylceramide (D’Angelo et al., 2007); Sec14/Nir2, which transports phosphatidylglycerol (Litvak et al., 2005); and OSBP, which might transport cholesterol (Perry and Ridgway, 2006). ER-to-Golgi transfer of ceramide and glucosylceramide by CERT and FAPP2 are of key functional importance because they are mandatory for the synthesis of sphingomyelin and glycosphingolipids by enzymes present only in the trans Golgi (D’Angelo et al., 2007; Hanada et al., 2003). The function of OSBP is less clear, and it has been proposed that OSBP and related proteins (ORP in mammals, Osh in yeast) either sense or transfer sterols (Beh et al., 2012; Beh and Rine, 2004; Mousley et al., 2012; Raychaudhuri et al., 2006; Wang et al., 2005). Nevertheless, the presence of OSBP in zones where CERT and FAPP2 act is intriguing because coenrichment in sphingolipids and cholesterol is a hallmark of late (trans Golgi, endosomes and PM), as opposed to early (ER and cis Golgi), compartments. OSBP and other lipid transporters might work in a coordinated manner to control the composition of the trans Golgi using the reservoir of lipids present in the ER (Lev, 2010; Peretti et al., 2008).

OSBP, FAPP2, and CERT share a similar domain organization consisting of an N-terminal pleckstrin homology (PH) domain, a central FFAT motif (two phenylalanines in an acidic track), and a C-terminal lipid transport domain (Lev, 2010; Levine, 2004). The PH domain detects two determinants of the trans Golgi: the phosphoinositide PI(4)P and the small G protein Arf1-GTP (Godi et al., 2004; Levine and Munro, 2002). On the other hand, the FFAT motif binds specifically the type II ER membrane protein VAP (Furuita et al., 2010; Kaiser et al., 2005; Loewen et al., 2003; Mikitova and Levine, 2012). Thus, OSBP, FAPP2, and CERT are equipped to bridge the ER and the Golgi, although this tethering activity has not been recapitulated in vitro.
their C-terminal regions, they are structurally diverse and correspond to domains that can extract specific lipid species; for example, the OSBP-related domain (ORD) in the case of OSBP (Im et al., 2005) or the START domain in the case of CERT (Kudo et al., 2008). Overall, and despite differences between these transporters, their domain organization suggests a similar division of labor between the PH-FFAT tandem, which might bridge the ER and the Golgi, and the C-terminal domain, which conveys specific lipids between the two membranes.

OSBP has recently been identified as the target of several anticancer and antiviral compounds (Anita et al., 2013; Burgett et al., 2011) pointing to its important role in cellular homeostasis. However, how OSBP and its relatives function at membrane interfaces is poorly understood. Here, we use a combination of reconstitutions on artificial membranes and cellular approaches to address the mechanism of membrane tethering and lipid transfer by OSBP, as well as the coupling between these activities. We show that the PH-FFAT region of OSBP bridges membranes containing the ER protein VAP-A to membranes containing PI(4)P or Arf1-GTP. Furthermore, membrane bridging directs sterol transfer by the ORD domain. Surprisingly, however, the ORD domain in turn controls membrane tethering by the PH-FFAT region. This feedback is due to two additional reactions. First, PI(4)P is transported by the ORD domain, which, like the yeast protein Osh4, acts as a sterol/PI(4)P exchanger (de Saint-Jean et al., 2011). Second, PI(4)P, once transported back from the Golgi to the ER, becomes accessible to the PI(4)P phosphatase Sac1, which, in contrast to a previous report (Stefan et al., 2011), preferentially hydrolyzes PI(4)P in cis rather than in trans. This first complete reconstitution of a lipid transporter at ER-Golgi contact sites suggests a general way to couple a specific lipid transfer reaction with the back transfer and hydrolysis of PI(4)P.

RESULTS

OSBP Contains a Potent Membrane Tethering Region but Is Predominantly Cytosolic

To determine the ability of OSBP to tether the ER to the Golgi, we first compared the localization of full-length OSBP and a construct encompassing the PH domain, the coiled-coil region and the FFAT motif (PH-FFAT; Figure 1A). Both constructs were tagged with mCherry and cotransfected with a GFP version of the ER receptor VAP-A (GFP-VAP-A) in HeLa cells.

Figure 1B and Figure S1A (available online) show that OSBP is predominantly cytosolic, with some faint staining of the ER network, which is marked by GFP-VAP-A. In agreement with previous observations (Ridgway et al., 1992), the addition of the oxysterol 25-OH induced a dramatic shift in the localization of OSBP, which translocated completely to perinuclear structures (Figure S1A). This shift was accompanied by the displacement of VAP-A, which no longer decorated the ER network, but instead concentrated in the same perinuclear regions as OSBP (Figure 1B).

In contrast to OSBP, PH-FFAT was never cytosolic: even in the absence of 25-OH, it localized to perinuclear regions where VAP-A, but not a mutant deficient in FFAT motif binding (KM > DD), concentrated (Figures 1C and S1A). These regions were also positive for the trans Golgi marker TGN-46 (Figure S1B), thereby suggestive of ER-Golgi MCSs.

To further characterize the perinuclear regions where VAP-A and the OSBP constructs concentrated, we used thin-section EM. We observed extensive ER/Golgi apposed regions in cells treated with 25-OH and expressing VAP-A and OSBP, as well as in untreated cells expressing VAP-A and PH-FFAT (Figures 1D, S1C, and S1D). In some cases, Golgi cisternae and vesicles were completely enwrapped by the ER and lipid bilayers were systematically paired at an even distance of ~20 nm. Fluorescence recovery after photobleaching (FRAP) indicated that VAP and OSBP or PH-FFAT exchanged very slowly from such zones (t1/2 > 5 min) compared to conditions where VAP and the OSBP constructs do not colocalize (Figures 1E and 1F).

We introduced point mutations in PH-FFAT to determine the molecular basis of membrane tethering. The R108L mutation, which prevents the interaction of the PH domain with PI(4)P (Levine and Munro, 2002), shifted the localization of PH-FFAT toward the ER (Figure S1E). Conversely, mutating the FFAT motif shifted the localization of PH-FFAT toward the Golgi (Figure S1E). Intriguingly, when the same mutations were introduced in full-length OSBP, the membrane association of this essentially soluble protein increased: the PH domain mutation promoted OSBP binding to the ER, whereas the FFAT motif mutation promoted OSBP binding to the Golgi (Figure S1F). Therefore, whereas the PH domain and the FFAT motif have additive effects for the localization of PH-FFAT at ER-Golgi MCS, they have a curious subtractive effect in the context of full-length OSBP.

These experiments indicate that the PH-FFAT tandem of OSBP is a very potent bridge to connect ER and Golgi membranes and that it does so by binding VAP-A via its FFAT motif and PI(4)P via its PH domain. However, in the context of full-length OSBP, the availability of the PH-FFAT tandem for membrane tethering is reduced and can be controlled by 25-OH, which targets the ORD.

Reconstitution of Membrane Tethering by the PH-FFAT Region of OSBP

We wished to recapitulate membrane tethering by the PH-FFAT tandem of OSBP in minimal systems. For this, we mixed purified PH-FFAT with two types of artificial liposomes (Figure 2A). Liposomes Lx contained a nickel lipid (DOGS-NiTA) to which the cytosolic domain of VAP-A was attached through a C-terminal polyhistidine tag; liposomes Ly contained PI(4)P and/or were covered by Arf1-GTP (Figure 2B). As such, Lx and Ly mimicked the ER and the Golgi apparatus, respectively.

We first used dynamic light scattering (DLS) to follow lipidome aggregation. Initially, the Lx + Ly mixture showed an apparent radius of ~70 nm. Upon PH-FFAT addition, the radius increased steadily, reaching values in the range of 500 nm within tens of minutes, suggesting massive lipidome aggregation (Figure 2C), which was confirmed by negative stained EM (Figure 2D). Control experiments indicated that aggregation required VAP-A on Lx and Arf1-GTP or PI(4)P on Ly. At low tether concentration, the effects of Arf1-GTP and PI(4)P were additive; at high tether concentration, efficient tethering was observed with either Arf1-GTP or PI(4)P (Figure 2C).
To further analyze PH-FFAT-mediated membrane tethering, we used artificial membranes that can be visualized by light microscopy, namely giant liposomes and bead-supported bilayers, which were labeled with red and green fluorescent lipids, respectively. PH-FFAT promoted the formation of extended contact zones between the giant liposomes, which carried VAP-A, and the bead-supported bilayers, which carried PI(4)P (Figure 2E; Movie S1). Contact zones were also observed when PH-FFAT was added to two populations of bead-supported bilayers with a similar segregation of membrane determinants (Figure 2F).
Full-Length OSBP Promotes Membrane Tethering In Vitro Independently of 25-OH

Using the same assays, we compared the tethering activity of purified full-length OSBP to that of PH-FFAT. Given our cellular observations (Figure 1), we expected OSBP to be much less active than PH-FFAT. Surprisingly, OSBP readily promoted the tethering of lipid membranes containing Arf1-GTP and/or PI(4)P to lipid membranes containing VAP-A. In fact, under all conditions tested, OSBP was at least as efficient as PH-FFAT (Figures S2 A–S2C). Furthermore, 25-OH, which promotes OSBP-mediated membrane tethering in cells, did not influence the tethering activity of OSBP in these reconstituted systems (Figure S2 D). Thus, whereas the tethering activities of PH-FFAT in vivo and in vitro matched well (compare Figures 1 C and 2), this was not the case for OSBP, which efficiently connected membranes in vitro (Figure S2), but not in cells, except in the presence of 25-OH (Figure 1B).

OSBP Changes the Cellular Distribution of Sterol and PI(4)P

Despite the lack of observable association of OSBP with organelles, we wondered whether the protein affects the distribution of its two putative lipid ligands: cholesterol and PI(4)P. To follow the distribution of PI(4)P, we used a GFP fusion of the PH domain of OSBP, which has been shown to be a fair reporter of PI(4)P levels at the Golgi (Levine and Munro, 2002). In control cells, the PI(4)P probe stained the Golgi apparatus and showed minor cytosolic distribution (Figure 3A). In contrast,
Figure 3. OSBP Affects the Cellular Distribution of Sterol and PI(4)P

(A) HeLa cells were transfected with a PI(4)P probe and various mCherry-OSBP constructs. The plot shows the amount of the PI(4)P probe in the perinuclear region. Measurements were performed on 50–80 cells for each condition using widefield fluorescence microscopy. Horizontal bars represent median values of the samples.
overexpression of OSBP caused a 3.5-fold drop in the Golgi staining by the probe (Figure 3A), suggesting a large decrease in the amount of PI(4)P in this organelle. Experiments using different forms of OSBP indicated that this decrease required a fully functional protein. First, mutations that compromise the interaction of OSBP with either VAP-A (mutation FF > AA in the FFAT motif) or PI(4)P (mutation R108L in the PH domain) made OSBP less efficient in reducing the PI(4)P level. Second, mutating two histidines and one lysine, which are strictly conserved in the ORD and essential for the extraction of PI(4)P by Osh4p (de Saint-Jean et al., 2011), abolished the drop in PI(4)P (Figures 3A and S3). PH-FFAT, which lacks the ORD, also did not affect the localization of the PI(4)P probe. Third, 25-OH reversed the effect of OSBP on PI(4)P levels, causing relocation of the PI(4)P probe to the Golgi (Figure 3A). Taken together these experiments suggest that OSBP controls the turnover of PI(4)P at the Golgi in a manner that involves its PH domain, its FFAT motif and its ORD. This effect requires the protein to not be blocked by 25-OH and involves the same residues that enable Osh4p to extract PI(4)P.

Visualizing cholesterol levels in cells is challenging due to its broad subcellular distribution and the lack of organelle-specific probes for this lipid. As a first step toward assessing the effect of OSBP on sterol trafficking, we pulse-labeled cells with dehydroergosterol (DHE), a naturally fluorescent analog of cholesterol, and chased it with an excess of cholesterol (Figure 3B). DHE can substitute for cholesterol because both have a very similar chemical structure in contrast to artificial fluorescent probes for this lipid. As a first step toward assessing the effect of OSBP on sterol trafficking, we pulse-labeled cells with dehydroergosterol (DHE), a naturally fluorescent analog of cholesterol, and chased it with an excess of cholesterol (Figure 3B). DHE can substitute for cholesterol because both have a very similar chemical structure in contrast to artificial fluorescent analogs (Mukherjee et al., 1998). In control cells, the exogenous addition of DHE caused immediate staining of the PM, followed within a few minutes of internal membranes and, after 1–2 hr, of lipid droplets (Figure 3B), indicative of a retrograde route from within a few minutes of internal membranes and, after 1–2 hr, of lipid droplets (Figure 3B), indicating that OSBP counteracts the retrograde traffic of sterols. However, they do not indicate at which location OSBP prevents this flux.

How can the inhibition of the flux of sterol from PM to lipid droplets be linked to the reduction of the Golgi pool of PI(4)P? The first effect leaves many possibilities for the exact site of OSBP action, whereas the second effect suggests that OSBP works at the ER-Golgi interface. Interestingly, mutations predicted to abolish PI(4)P binding (Figure S3A) prevented OSBP from counteracting the accumulation of DHE in lipid droplets (Figure 3B), suggesting that the two activities of OSBP are coupled.

Cholesterol is esterified and incorporated into lipid droplets when its level at the ER is high. Therefore, we hypothesized that OSBP exports sterol out of the ER at the expense of sterol incorporation into lipid droplets and in a manner that “consumes” PI(4)P at the Golgi. Specifically, OSBP might exchange the two lipids at the ER-Golgi interface. Measuring such an exchange in vivo and in the confined environment of Golgi/ER MCSs was, however, not possible. To determine if OSBP acts on the pool of cholesterol present in the ER, we used an alternative strategy: we lowered the level of cholesterol at the ER using lovastatin. As shown in Figures 3C and S3B, this treatment protected the pool of PI(4)P at the Golgi from being consumed by OSBP, which became trapped at the ER-Golgi interface. We concluded that OSBP controls the balance between sterol at the ER and PI(4)P at the Golgi.

Altogether, the experiments of Figures 3 and S3 suggest that the apparent cytosolic localization of OSBP is deceptive and masks an ephemeral interaction with the ER and the Golgi. In the case of PH-FFAT, we observe stable tethering because this construct acts solely as an ER-Golgi bridge. In contrast, OSBP via its ORD displaces PI(4)P from the Golgi (Figure 3A). Because PI(4)P contributes to the Golgi attachment of OSBP (via the PH domain), its displacement should eventually interfere with membrane tethering. In line with this hypothesis, OSBP mutants predicted to be deficient in PI(4)P transfer not only preserved the Golgi localization of the PI(4)P probe (Figure 3A) but also remained concentrated at ER-Golgi MCSs (Figure 3D). Therefore, OSBP might be controlled by a negative feedback loop whereby membrane tethering promotes sterol/PI(4)P exchange, which in turn impairs membrane tethering.

**OSBP Is Autoinhibited for Sterol Transport**

As a first step toward testing this feedback model, we assessed the lipid transport activity of OSBP in vitro. OSBP did not accelerate the exchange of the sterol DHE between liposomes that contained no determinants to promote binding of the PH-FFAT region (Figure 4A, red trace). We then submitted OSBP to limited proteolysis and observed a dramatic acceleration of DHE transfer, which paralleled the first steps of OSBP cleavage (Figure 4A, red traces). These results suggest that OSBP is autoinhibited for sterol exchange.

We analyzed the products of the proteolysis using N-terminal sequencing and gel filtration chromatography (Figures 4B, 4C, and S4). This analysis revealed a few major products: (1) a 43 kDa fragment encompassing the PH domain and the FFAT motif, (2) a 35 kDa fragment derived from the previous one but lacking the FFAT motif, (3) various fragments of 30–35 kDa corresponding to the N-terminal part of the ORD, and (4) a 20 kDa fragment corresponding to the remaining region of the ORD. In

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gel filtration chromatography, the first two products eluted at an apparent molecular weight (MW) of about ~80 kDa, suggesting dimerization through coiled-coil regions between the PH domain and the FFAT motif. The ORD fragments (30–35 and 20 kDa) coeluted at a MW of 50 kDa, suggesting that cleavage of the ORD at position R669-G670 does not promote separation of the domain parts. Membrane tethering activity was present in the first gel-filtration peak, whereas DHE exchange activity was present predominantly in the second peak (Figure 4C). This analysis confirms the division of labor in OSBP: the PH-FFAT tandem mediates membrane tethering and the ORD mediates lipid transport.

Sterol Transfer by OSBP Requires VAP-A and Is Facilitated by Arf1-GTP

To determine the mechanism by which OSBP becomes active for sterol transfer, we included various combinations of VAP-A, Arf1-GTP, and PI(4)P on the liposomes used for the DHE assay. The red trace in Figure 5A shows a representative time course of DHE transfer in an experiment where VAP-A was attached to L_a and Arf1-GTP was attached to L_b. Under such conditions, the rate of DHE transfer catalyzed by $10^{-7}$ M OSBP was very fast and close to that observed with $10^{-3}$ M methyl-$\beta$-cyclodextrin (MCD; Figure 5A, gray trace), indicating that OSBP has a 10,000-fold higher exchange activity than the drug.

Removing VAP-A from L_a abolished OSBP-catalyzed DHE transfer, whereas removing Arf1-GTP from L_b reduced the rate ~3-fold to 5-fold (Figures 5A–5C). Therefore, the interaction of OSBP with VAP-A is mandatory for sterol exchange, whereas the interaction with Arf1-GTP is helpful, but not strictly required. Titration experiments gave a half-stimulatory effect of 300 nM for VAP-A and of 100 nM for Arf1-GTP (Figures 5B and 5C). From the amounts of DHE and OSBP present (11 µM and 100 nM, respectively), we calculated that each cycle of sterol transfer takes about 2–3 s ($k = 0.3–0.5$ s$^{-1}$) under optimal conditions (Figures 5B and 5C). 25-OH blocked OSBP-catalyzed transfer of DHE with a $K_i$ of 50 nM (Figure 5D), a value compatible with the reported affinity of 25-OH for OSBP ($\sim 10$ nM) (Ridgway et al., 1992).

To better characterize the mechanism of OSBP activation, we conducted experiments with soluble forms of VAP-A and Arf1-GTP. VAP-A retained a substantial stimulatory effect
even in the absence of nickel lipids (Figure S5A), whereas soluble Arf1-GTP had no significant effect (Figure S5B). These observations suggest that VAP-A not only acts as a membrane anchor but also affects the conformation of OSBP, whereas Arf1-GTP acts primarily as a membrane anchor. The importance of the VAP-FFAT interaction in OSBP-mediated DHE exchange was underscored by the effect of a peptide corresponding to the FFAT motif, which completely blocked DHE transfer ($K_i = 0.82 \pm 0.15 \, \mu M$, Figure 5E). As for the stimulatory effect of Arf1-GTP, it disappeared when the small G protein was attached to $L_a$ instead of $L_b$ (Figure S5B), suggesting that the membrane determinants of OSBP should be present on different liposomes to promote lipid exchange.

**Complex Effect of PI(4)P on Sterol Transfer by OSBP**

In membrane tethering assays, Arf1-GTP and PI(4)P had interchangeable roles, both promoting lipid aggregation by PH-FFAT (Figure 2C). In contrast, the effects of PI(4)P and Arf1-GTP on DHE transfer by OSBP were very different. In the presence of PI(4)P in $L_a$, the kinetics of DHE transfer from $L_a$ to $L_b$ showed a biphasic shape (Figures 6A and S6A). During the first minute, DHE transfer occurred rapidly. Then, it abruptly slowed down. Considering that, under our conditions, the overall transfer of DHE from $L_a$ to $L_b$ requires about 50 cycles of OSBP-mediated DHE transfer, this observation suggests that PI(4)P becomes inhibitory after OSBP has undergone a few cycles (Figure S6B).

To explain these observations we envisaged that, through a mechanism akin to Osh4p (de Saint-Jean et al., 2011), the ORD of OSBP could extract PI(4)P from $L_b$ and transfer it to $L_a$. As such, PI(4)P should gradually inhibit DHE transfer by two effects: competition with DHE on $L_a$ for PI(4)P extraction and inhibition of OSBP activity as the PI(4)P transfer reaction proceeds, this observation hints at a possible inhibition of OSBP activity as the PI(4)P transfer reaction proceeds, an effect reminiscent to what we observed for DHE transfer (Figure 6A).

**Control of OSBP-Induced Membrane Tethering by the PI(4)P Phosphatase Sac1**

We reasoned that OSBP, by being able to transfer PI(4)P in a VAP-A-dependent manner, should, in the context of ER-Golgi MCS, promote the transfer of PI(4)P from the trans Golgi, which is rich in this phosphoinositide, to the ER. There, PI(4)P could be hydrolyzed by the integral ER membrane protein Sac1 (Kim et al., 2013). This sequence of events would explain why OSBP reduces the amount of PI(4)P at the Golgi (Figure 3A) and why the combined effects of the PH domain and the FFAT motif are subtractive for the localization of full-length OSBP (Figure S1D): membrane tethering is followed by the transfer and degradation of PI(4)P, leading to membrane detachment of OSBP.

To test this hypothesis, we first used small interfering RNA (siRNA) against Sac1 in cells expressing OSBP and VAP-A. Strikingly, knocking down Sac1 completely shifted the localization of OSBP from an essentially cytosolic distribution to a perinuclear one where VAP-A colocalized (Figure 7A). Thus, in the cell,
OSBP has the ability to tether ER and Golgi membranes, but Sac1 counteracts this activity.

Next, we addressed the effect of Sac1 on the tethering activity of OSBP in vitro. For this, we mixed L\textsubscript{a} and L\textsubscript{b} liposomes to imitate the ER/trans-Golgi interface and monitored liposome aggregation by DLS. Liposomes L\textsubscript{b} were coated with both VAP-A and with Sac1 via their C-terminal polyhistidine tags, which replaced the juxtamembrane regions of these ER proteins. L\textsubscript{b} contained PI(4)P. Thus, PI(4)P and Sac1 were initially present in different membranes, i.e., in a \textit{trans} orientation. These experiments revealed a marked difference between full-length OSBP and PH-FFAT (Figure 7B): membrane tethering induced by OSBP strongly diminished in the presence of 5 to 10 nM Sac1, whereas membrane tethering induced by PH-FFAT was more resistant, diminishing significantly only at Sac1 concentration above 15 nM.
The experiments shown in Figures 7A and 7B suggest that Sac1 is the missing link for reconciling the in vitro and cellular observations. Adding Sac1 to liposome reconstitutions recapitulated the differences in the tethering activities of OSBP and PH-FFAT that were obvious in cellular experiments (Figures 1B and 1C). Conversely, depleting Sac1 from cells by siRNA rendered OSBP as efficient as PH-FFAT in Golgi-ER tethering, in agreement with our initial lipidosome reconstitutions where Sac1 was absent (Figures 2 and S2).

Sac1 Preferentially Hydrolyzes PI(4)P in cis

In Sac1, the catalytic site is cytosolic and is connected to the transmembrane domain with a putative 17 nm flexible linker. It has been proposed that this feature allows Sac1 to hydrolyze PI(4)P in trans, i.e., when PI(4)P is present in an apposed membrane (Stefan et al., 2011). However, we noted that the results of our tethering assay were more consistent with a cis activity of Sac1. In this assay, PI(4)P was initially present in trans from Sac1, yet Sac1 disrupted more efficiently membrane tethering by OSBP, which should transfer PI(4)P between the liposomes, than by PH-FFAT, which should leave the trans segregation unchanged (Figure 7B).

To directly address the optimal orientation of PI(4)P for Sac1-mediated hydrolysis, we monitored phosphate release using a green malachite assay. Again, Lb and La liposomes were mixed; the former contained DOGS-NIANTA to bind Sac1 and VAP-A, and the latter contained PI(4)P. PH-FFAT, which stably bridges these liposomes, thereby imposing a trans segregation of Sac1 and PI(4)P, did not promote PI(4)P hydrolysis (Figure 7C, gray circles). In contrast, OSBP, which transfers PI(4)P from Lb to La, stimulated PI(4)P hydrolysis in a VAP-A-dependent manner (Figure 7D). Control experiment in which PI(4)P was directly included in Lb demonstrated that Sac1 readily hydrolyzed PI(4)P in cis (Figure 7C, pink circles).

PI(4)P Hydrolysis by Sac1 Relieves OSBP Blockage

As aforementioned, the gradual inhibitory effect of PI(4)P on OSBP-catalyzed DHE transfer probably arises from PI(4)P backward transfer to the ER-like membrane (liposomes Lb), where it should antagonize DHE extraction. We predicted that Sac1, by hydrolyzing PI(4)P in cis, should relieve this inhibition. We thus repeated the DHE transport experiment in the presence of Sac1, which was attached to Lb together with VAP-A. As before, Lb contained DHE, whereas Ls contained PI(4)P. Strikingly, Sac1 had no effect on the initial fast phase of DHE transfer but strongly accelerated the second phase in a concentration-dependent manner (Figure 7E), except when 25-OH was present (Figure S7A). This observation demonstrates that VAP-A, OSBP, and Sac1 act in a sequential manner: VAP-A directs the orientation of OSBP, OSBP exchanges sterol for PI(4)P, and Sac1 specifically hydrolyses PI(4)P when this lipid becomes in cis. Another cycle of sterol/PI(4)P exchange can then resume until the pool of PI(4)P left in Lb is consumed.

DISCUSSION

Our work suggests a minimal model for how three proteins—the lipid transporter OSBP, the general ER receptor VAP-A, and the PI(4)P phosphatase Sac1—coordinate their activities to tether membranes, to promote the specific exchange of lipids between them and to make these events self-regulated over time. Our model includes four steps (Figure 7F), each of which we have reconstituted with minimal components: (1) membrane tethering, (2) forward sterol transfer, (3) backward PI(4)P transfer, and (4) PI(4)P hydrolysis. This last reaction makes the cycle irreversible and, when PI(4)P becomes limiting, acts as a timer to stop membrane pairing. In the following paragraphs, we discuss each step separately, and then envisage their coordination.

Step 1: Membrane Tethering

The PH-FFAT tandem of OSBP is an efficient membrane tether. In cells, it caused massive pairing of Golgi membranes with the ER (Figures 1C and 1D). In vitro, it promoted the aggregation of membranes in an asymmetric manner according to specific determinants (Figure 2): on the one hand, VAP-A, which interacts with the FFAT motif (Furuita et al., 2010; Kaiser et al., 2005); on the other hand, PI(4)P or Arf1-GTP, which interact with the PH domain (Godi et al., 2004; Levine and Munro, 2002). The next step will be to determine the structure of the entire PH-FFAT module. The presence of coiled coils between the PH domain and the FFAT motif suggests a dimeric rod-like structure (Figures S7B and S7C). This architecture, which is found in many tethering molecules, combines two advantages: it imposes a tether length and allows multivalent interactions with the membrane (Dumas et al., 2001). The predicted coiled coils of OSBP would give a rod of about 11 nm interrupted by an elbow. When the sizes of the other domains are taken into account, this length is compatible with the distance between tethered membranes observed in cells (~20 nm; Figures 1D and S7C).

Step 2: Sterol Transfer

The ability of OSBP to bind sterols and oxysterols is established (Ridgway et al., 1992). More debated is OSBP’s function in sterol transfer. It has been argued that the rate of sterol transfer by Osh/ ORP proteins is not fast enough to account for the quick redistribution of cholesterol between organelles (Beh et al., 2012). Using assays with high temporal resolution, we report a maximal turnover rate of 0.5 s⁻¹, i.e., 30 DHE transferred per OSBP and per minute (Figure 5C). This value is much faster than what has been measured in vitro for the transfer of ceramide by CERT, which is unambiguously a ceramide transfer protein (Hanada et al., 2003; Kawano et al., 2006). Therefore, OSBP seems intrinsically adapted to quickly transfer sterols between the ER and the Golgi.

Step 3: PI(4)P Transfer

We previously demonstrated that Osh4p exchanges sterol for PI(4)P between artificial membranes and resolved the structure of Osh4p in complex with PI(4)P (de Saint-Jean et al., 2011). Our experiments indicate that OSBP is also capable of transferring PI(4)P between membranes (Figure 6C). Furthermore, mutating conserved residues that allow Osh4p to extract PI(4)P prevented OSBP from reducing the PI(4)P level at the Golgi (Figure 3A). Thus, the ORD might be a general fold to exchange specific lipids with PI(4)P.
Figure 7. Sac1 Regulates OSBP-Mediated Membrane Tethering and Lipid Exchange

(A) HeLa cells expressing GFP-VAP-A and mCherry-OSBP were transfected with scrambled siRNA (control) or Sac1 siRNA and incubated for 24 hr in growth medium. OSBP and VAP-A moved to perinuclear areas in Sac1-silenced cells.

(B) Liposome tethering assay. The sample contained liposomes La (12.5 μM lipids, 2% DOGS-NNTA), Lb (12.5 μM lipids, 2% PI(4)P), 1 μM VAP, and increasing concentrations of Sac1. At t = 100 s, PH-FFAT (0.2 μM, left) or OSBP (0.2 μM, right) was added. Liposome aggregation was followed by DLS. Sac1 drastically reduced liposome tethering induced by OSBP, but not by PH-FFAT.

(legend continued on next page)
Step 4: PI(4)P Hydrolysis
PI(4)P is synthesized by specific PI-4-kinases at the Golgi and the PM, yet its phosphatase, Sac1, is an integral ER membrane protein (Kim et al., 2013). Recently, a mechanism has been proposed to solve this paradox: that Sac1 hydrolyses PI(4)P in trans (Stefan et al., 2011). All our results point to the opposite mechanism, whereby Sac1 hydrolyzes PI(4)P in cis, i.e., in the same membrane. PH-FFAT, which bridges very efficiently ER to Golgi membranes, thereby positioning Sac1 in trans toward PI(4)P, does not affect the cellular distribution of PI(4)P (Figure 3A). Recapitulating the trans segregation between Sac1 and PI(4)P using two liposome populations and PH-FFAT as a tether also does not promote PI(4)P hydrolysis (Figure 7C). In contrast, full-length OSBP, which transfers PI(4)P from trans to cis membrane, allows PI(4)P hydrolysis by Sac1 (Figure 7D). Thus, in the context of ER-Golgi MCSs promoted by OSBP, Sac1 works in cis, although we cannot exclude that it acts in trans in other circumstances. In yeast, Sac1 deletion causes PI(4)P to redistribute from the Golgi to other membranes, including the ER (Faulhammer et al., 2007; Roy and Levine, 2004), an observation that fits with a sequence of events in which PI(4)P hydrolysis occurs after its transfer from the Golgi to the ER.

Coordination between Membrane Tethering, Lipid Exchange, and PI(4)P Hydrolysis
PI(4)P hydrolysis by Sac1 allows multiple round of sterol transfer by OSBP in the presence of VAP-A (Figure 7E). This result demonstrates that OSBP functions in a cyclic manner by using the metabolic energy of PI(4)P and implies tight coupling between all steps of the cycle.

Conditions for efficient sterol transfer (step 2) parallel conditions for efficient tethering (step 1) (Figures 2C and 5A), suggesting that OSBP simultaneously contacts the ER and the Golgi during at least one step of its cycle. In the confined space between two apposed membranes, the ORD must move within distances ~four times larger than its own size (Figure S7C), implying a fascinating gymnastic. It seems that the PH-FFAT tandem not only restricts the diffusion of the ORD, but also plays an active role. First, the interaction between VAP-A and the FFAT motif is mandatory for lipid transfer by the ORD (Figures 5 and 6C). Second, sequence comparison between ORP/Osh proteins shows that the FFAT motif is localized at fixed distance from the ORD N terminus, which itself acts as a lid to control accessibility to the lipid-binding pocket (Im et al., 2005). Thus, the FFAT-VAP-A interaction is probably critical to unlock the ORD.

When OSBP harbors mutations preventing the ORD from displacing PI(4)P form the Golgi (Figure 3C). Therefore, forward transport of sterol by OSBP at MCSs seems coupled to backward transport of PI(4)P. Direct demonstration of this coupling will require detecting simultaneously the exchange of these two lipids at MCSs. This task is very challenging in vivo, as it implies following the displacement of lipids within distances of tens of nanometers. In vitro, however, the fact that extended MCSs with a simple geometry can be recapitulated (Figure 2E; Movie S1) suggests possibilities for visualizing lipid movements using fluorescent analogs and probes.

Whereas the causality between membrane tethering (step 1), sterol transfer (step 2), and PI(4)P back-exchange (step 3) could be anticipated given the architecture of OSBP and the similarity between its ORD and Osh4p (Mesmin et al., 2013), the PI(4)P hydrolysis step (step 4) after retrograde transfer of PI(4)P, seems counterintuitive: by transferring PI(4)P to the ER where PI(4)P is hydrolyzed by Sac1, OSBP “shoots itself in the foot,” eliminating a determinant that contributes to its own attachment. However, this reasoning is valid when the level of PI(4)P at the Golgi becomes limiting, i.e., when the trans Golgi looses its identity. When PI-4-kinases continuously regenerate PI(4)P at the trans Golgi, OSBP can cycle many times provided that Sac1 hydrolyses PI(4)P at the ER. For such an unidirectional cycle to function, it is essential that PI(4)P hydrolysis occurs in cis; if Sac1 acted in trans, it would burn PI(4)P prematurely, i.e., before sterol transfer. The OSBP cycle relies fundamentally on the segregation of VAP-A, Sac1, and PI4-kinases between the ER and the Golgi, which allows PI(4)P hydrolysis at the ER to provide the energy for sterol transfer. Interestingly, similar cycles may occur at other MCS: recent observations indicate that PI-4-kinases contribute to sterol enrichment at the PM where other ORP/Osh proteins act (Nakatsu et al., 2012). Because PI(4)P is present not only at the Golgi but also at the PM, further work is needed to determine the combinations of membrane determinants that direct the specific targeting of OSBP and its relatives to different membranes.

We note that the apparent coupling between various lipid transfer reactions at ER-Golgi MCSs (Peretti et al., 2008; Perry and Ridgway, 2006) can be explained on the basis of the OSBP cycle. FAPP2 and CERT rely on the same membrane determinants as OSBP. By controlling the amount of PI(4)P in the target membrane, OSBP could set the tempo for the delivery of precursors of complex lipids to the trans Golgi, thereby insuring that the concentrations of cholesterol and sphingolipids increase in parallel along the ER-Golgi interface.

CONCLUSIONS
The sequence of reactions that we demonstrate for OSBP invites a comparison with ion pumps. Through the use of metabolic

(C and D) Sac1 phosphatase assay. To test the cis orientation, Sac1 (50 nM) was added to liposomes L a containing 2 mol % DOGS-NiNTA and 20 mol % PI(4)P. To test the trans orientation, Sac1 (50 nM) was added to a mixture of L a containing 2 mol % DOGS-NiNTA and L b containing 20 mol % PI(4)P. L a and L b were bridged with 0.2 μM PH-FFAT (C) or OSBP (D) in the presence of VAP (1 μM). When PI(4)P was initially present in trans, PI(4)P hydrolysis occurred in an OSBP- and VAP-A-dependent manner. Data represent average (±SEM) from 3 to 6 independent experiments.

(E) Sac1 stimulates DHE transfer. The experimental conditions were the same as in Figure 6A except that increasing amounts of Sac1 were added. L a contained 1 μM VAP. L a contained 10% PI(4)P. Sac1 relieves the inhibition caused by PI(4)P after a few rounds of lipid transfer.

(F) Model of the OSBP cycle. For simplicity, only one OSBP monomer is shown. See also Figure S7.
energy (phosphorylation and dephosphorylation of a catalytic residue), ions pumps cycle up to 100 times/s to maintain an asymmetric distribution of ions across lipid membranes (Palmgren and Nissen, 2011). Functionally, the OSBP cycle seems analogous, except that it helps to maintain an asymmetric distribution of lipids across a hydrophilic barrier using the metabolic energy of phosphoinositides.

**EXPERIMENTAL PROCEDURES**

Protein expression, purification, limited proteolysis, and gel filtration, as well as cell-culture conditions, transfections, and imaging, are described in the Extended Experimental Procedures.

**Liposomes**

The default composition of liposomes La and Lb was egg PC/brain PS/DOGS-NINTA (93/5/2 mol %) and egg PC/liver PE brain PS/liver PI (66/19/5/10 mol %), respectively. Depending on the assay, DHE, Dansyl-PE, Rhodamine-PE, Oregon green-DHPE, and/or Pl(4)P were included in the lipid composition as indicated. See the Extended Experimental Procedures for additional details.

**Liposome Aggregation**

Liposome aggregation was followed by DLS on mixtures containing liposomes La and with the indicated amounts of VAP-A and OSBP or PH-FFAT. The reaction was initiated by the addition of 250 nM PH-FFAT or 250 nM OSBP and was followed at 30°C by acquiring an autocorrelation curve every 10 s. Data were analyzed assuming a single Gaussian distribution, thus giving an average radius. See the Extended Experimental Procedures for additional details.

**Tethering Assay with Giant Liposomes and Templates**

Giant liposomes (type La + 2% Rhodamine-PE) were prepared by spontaneous formation. Bead-supported bilayers were prepared by incubating 5 μm silica beads for 30 min with liposomes (type La, 200 μM, 2% Oregon green-DHPE). Tethering was initiated by adding the giant liposomes with 250 nM VAP-A to the bead-supported bilayers (5 μM lipids) in the presence of 250 nM PH-FFAT or OSBP. Fluorescence images were acquired with a confocal microscope using a 63× objective. See the Extended Experimental Procedures for details.

**DHE Microscopy**

DHE imaging was carried out using a Leica DMIRBE microscope equipped with an Andor iXon3 blue-optimized EMCCD camera and Semrock BrightLine fluorescence filters (320/40 nm bandpass filter, 347 nm dichroic beamsplitter, and 390/40 nm bandpass filter). Images were acquired using a 100×/1.3 oil objective. See the Extended Experimental Procedures for additional details.

**DHE and Pl(4)P Transfer Assays**

For DHE transfer, the sample initially contained liposomes Lb (25 μM lipids) + VAP-A (200–250 nM) + Sac1, and liposomes La (25 μM lipids) ± 200 nM Arf1-GTP ± 2 mol % Pl(4)P. Aggregation was initiated by the addition of 100–250 nM PH-FFAT or 250 nM OSBP and was followed at 30°C by acquiring an autocorrelation curve every 10 s. Data were analyzed assuming a single Gaussian distribution, thus giving an average radius. See the Extended Experimental Procedures for additional details.

**Sac1 Pl(4)P Hydrolysis Assay**

Liposomes Lb were incubated with Sac1 and with the indicated amounts of VAP-A and OSBP or PH-FFAT. The reaction was initiated by the addition of liposomes La (20% Pl(4)P). At the indicated times, an aliquot was withdrawn, mixed with 50 mM NEM, and supplemented with malachite green reagent for phosphate quantification. For the experiment in the cis orientation, Lb were supplemented with 20% Pl(4)P. See the Extended Experimental Procedures for additional details.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes Extended Experimental Procedures, seven figures, and one movie and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2013.09.056.

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**REFERENCES**


EXTENDED EXPERIMENTAL PROCEDURES

Reagents
Full-length and fragments sequence of human OSBP1 were PCR amplified from the ImaGENES clone IRAUp969H0566D. Methyl-β-cyclodextrin, N-ethylmaleimide (NEM), the oxysterol 25-hydroxycholesterol (25-OH), Phenylarsine Oxide (PAO) and lovastatin were purchased from Sigma-Aldrich. The SYPRO-Orange protein gel stain and the neutral lipid stain LipidTOX green were from Invitrogen. Rabbit anti-Sac1 and anti beta-actin were purchased from Thermo Fisher Scientific and Epitomics, respectively.

OSBP Expression and Purification
Full-length, C-terminal His-tagged OSBP1 was expressed and purified from baculovirus-infected Sf9 cells. DNA was amplified by PCR and cloned into pENTR/D-Topo (Invitrogen), then modified by insertion of a thrombin site sequence in frame at the end of the OSBP sequence. Inserts were verified by sequencing, inserted into linearized BaculoDirect (C-terminal V5 and His tagged) by recombination, and transfected into Sf9 cells. For large-scale expression and purification, Sf9 cells were infected at an MOI of 0.1 for 1 hr at room temperature, suspended in 500–1000 ml of Sf-900 II media with 1.5% FCS and incubated with shaking at 27°C. After 72 hr, cells were collected by centrifugation at 300 × g for 10 min and stored at −20°C. Pellets were resuspended in 1/50 volume of lysis buffer (20 mM Tris pH 7.5, 300 mM NaCl, 20 mM imidazole, EDTA-free protease inhibitors and phosphatase inhibitors) and lysed with Dounce homogenizer. After centrifugation (50 000 × g, 30 min, 4°C) OSBP from the supernatant was adsorbed on an Ni-NTA metal-affinity resin (QIAGEN), submitted to 3 washes with lysis buffer supplemented with 800, 500, and 300 mM NaCl, respectively, and then eluted with 0.25 M imidazole-containing buffer. OSBP fractions were pooled, concentrated on Amicon Ultra (cut-off 30 000 kDa), and then purified on a Superdex 200 HR10/30 column (GE Healthcare). OSBP fractions were pooled, concentrated and then submitted to thrombin cleavage for 1 hr at 25°C to eliminate the C-terminal His tag. The purified protein was stored at −80°C in presence of 10% glycerol.

Expression and Purification of PH-FFAT, FAPP1-PH Domain, VAP-A, and Sac1
PH-FFAT ( = OSBP 76-408) was sub-cloned into pGEX-4T3 vector (GE Healthcare) for expression as a GST fusion protein in *E. coli*. FAPP1-PH domain (kindly provided by Franck Perez, Institut Curie) was sub-cloned into pGEX-4T3 and was modified by point mutagenesis (C37S/C94S/ T13C/T100S) for specific NBD labeling of C13. A linker insertion (NGNLSSLSA) upstream of the PH sequence allows thrombin cleavage of the GST fusion as well as recovery of the isolated FAPP1-PH domain. Human VAP-A (8-212) and yeast Sac1 (1-522) fragments were sub-cloned into pET-21b for expression as C-terminal His-tag proteins in *E. coli*. In both cases the position of the C-terminal tag corresponds to beginning of the C-ter transmembrane region. As such, the constructs should be positioned on DOGS-NNTA liposomes in a manner similar to the authentic membrane proteins. GST fusion proteins were purified on glutathione Sepharose beads (GE Healthcare), cleaved by thrombin, and His-tag fusion proteins were purified on Ni-NTA beads (QIAGEN). Myristoylated Arf1 was purified from *E. coli* coexpressing bovine Arf1 and N-myrissotransferase through ammonium sulfate precipitation, DEAE chromatography, and MonoS chromatography as described (Franco et al., 1995). Purified recombinant proteins were stored with 10% glycerol at −80°C. NBD labeling of FAPP1-PH domain was performed as described (de Saint-Jean et al., 2011).

N-Terminal Sequence Determination
OSBP fragments obtained from 250 μg of purified OSBP after trypsin proteolysis were resolved on a Superose 12 gel filtration column. Fragments separated in two peaks. The protein material from each peak was pooled, concentrated and separated on SDS-PAGE before transfer onto PVDF membrane. Two main fragments of 43 kDa and 35 kDa were obtained from peak 1, whereas three other main fragments of 35 kDa, 33 kDa and 20 kDa were obtained from peak 2. Isolated protein bands were extracted from the membrane and sent for sequencing at the Plate-forme Protéomique de l’Institut de Microbiologie de la Méditerranée (Marseille Protéomique, IBiSA, CNRS).

Cell Culture and Transfection
HeLa cells were grown in DMEM (Invitrogen) supplemented with 10% fetal calf serum (FCS) and antibiotics (Zell Shield, Minerva biosabs). For protein expression, cells were transfected with Lipofectamine 2000 reagent for 18 to 24 hr. OSBP with mCherry at the N terminus was constructed in a pmCherry-C1 vector. The PH-FFAT tandem (OSBP[76-408]) with mCherry at the C terminus was constructed in a pmCherry-N1 vector. Alternatively, PH-FFAT was subcloned in a PTunerC-GFP vector (Clontech Laboratories). Mutants were generated with the QuikChange Lightning site-directed mutagenesis kit (Agilent Technologies). The PI(4)P probe (OSBP PH domain with GFP at the N terminus) was cloned in a pEGFP-C1 vector. VAP-A and VAP-A K94D/M96D (VAP-A KM > DD) with N-terminal GFP were constructed in a pEGFP-C1 vector (gift from Fabien Alpy, Ilkirsch). For Sac1 silencing, Sac1 siRNA (Santa Cruz Biotechnology) was transfected in HeLa cells with Lipofectamine 2000 for 24 hr. Control siRNA were purchased from Santa Cruz. For confocal fluorescence microscopy, cells were plated on fibronectin-coated glass coverslips. For wide-field imaging, cells were plated on ibiTreat μ-Slides 8 well (Ibidi).
Live Cell Imaging, FRAP Assay, and Data Acquisition

For live cell imaging and FRAP assay, HeLa cells were maintained at 37°C in growth medium supplemented with 2.5 mM HEPES, or in medium A (NaCl 150 mM, HEPES 20 mM pH 7.55, CaCl2 1 mM, KCl 5 mM, MgCl2 1 mM) supplemented with 2 mg/ml glucose in the case of DHE imaging. Confocal fluorescence microscopy and FRAP assays were carried out using a Nikon Eclipse Ti inverted microscope equipped with an UltraVIEW VoX spinning disc imaging system (PerkinElmer) driven by Velocity software. Images were acquired using an 100 x oil-immersion objective (Nikon CFI Plan Apochromat 100 x/1.4). For FRAP experiments, photo-bleaching was performed on circular areas of 3.5 µm² within cell perinuclear regions. Fluorescence in these areas was then recorded every 0.5 s.

Wide-field microscopy imaging of the Pl(4)P probe was carried out using a Plan-FLUAR 100 x/1.45 oil objective on an Axiovert 200 M microscope (Zeiss) equipped with a CoolSNAP HQ CCD camera (Roper Scientific). Image acquisition and analysis was performed with MetaMorph software (Molecular Devices). To determine the fraction of the Pl(4)P probe in the perinuclear region, images were first background corrected, then two fluorescence intensity thresholds were applied to the images. A low threshold was set to define the total area of the cells, whereas a high threshold was set to identify the bright, Pl(4)P probe-labeled, perinuclear regions. The fluorescence intensity ratio obtained from the two threshold values gives Pl(4)P probe amounts in the perinuclear region of the cells. Alternatively, imaging of the Pl(4)P probe was carried out using confocal microscopy. In this case, the Golgi/cytosol Pl(4)P probe fluorescence ratio per cell was determined by dividing the mean fluorescence intensity obtained from three regions of 3 µm² within the Golgi area to the mean fluorescence intensity obtained from three regions of 3 µm² within the cytosol area.

Wide-field microscopy for DHE imaging was carried out using a Leica DMIRBE microscope equipped with an Andor iXon3 blue-optimized EMCCD camera driven by Solis software (Andor). Cells were incubated with DHE in complex with MCD (1:5 ratio) for one min at 37°C and washed 3 times in medium A. Cells were further incubated in growth medium containing cholesterol in complex with MCD for 2 hr before imaging. DHE was imaged using Semrock BrightLine fluorescence filters (320/40 nm bandpass filter, 347 nm dichroic beamsplitter, and 390/40 nm bandpass filter). Images were acquired using a Leica HCX APO 100 x/1.3 oil U-V-I objective.

FFAT Peptide

Peptide synthesis was ordered from Proteogenix (Oberhausbergen, France). The sequence (WCGKGDMDSDEFDDEAPEITM PENLGH) reproduces the FFAT motif sequence of OSBP with an extra N-terminal tryptophan for accurate concentration determination by UV spectroscopy.

Lipids

Egg-PC, brain PS, brain Pl(4)P, brain Pl(4,5)P2, liver PI, liver PE, Dansyl-PE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(5-dimethylamino-1-naphthalenesulfonyl)), Rhodamine-PE (1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine-N-(lissamine rhodamine B sulfonyl)), and DOGS-NiNTA (1,2-dioleoyl-sn-glycero-3-[N(5-(amino-1-carboxyptyl)limidodiaceit acid)succinyl] (nickel salt)] were obtained from Avanti Polar Lipids. PI(3)P (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(5-diethylaminoo-1-naphthalenesulfonyl)) and PI(4,5)P2 (1,2-dioleoyl-sn-glycerol-3-phosphoethanolamine-N-(5-diethylaminoo-1-naphthalenesulfonyl)) were obtained from Avanti Polar Lipids. PI(3)P (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-(5-diethylaminoo-1-naphthalenesulfonyl)) was from Avanti Polar Lipids. The concentration of DHE in the stock solution in methanol was carefully determined by UV spectroscopy.

Liposome Preparation

Lipids in stock solutions in chloroform were mixed at the desired molar ratio, and the solvent was removed in a rotary evaporator. The lipid film was hydrated in 50 mM HEPES pH 7.2 and 120 mM potassium acetate (HK buffer) to give a suspension of large multilamellar lipid film. Liposomes were first background corrected, then two fluorescence intensity thresholds were applied to the images. A low threshold was set to define the total area of the cells, whereas a high threshold was set to identify the bright, Pl(4)P probe-labeled, perinuclear regions. The fluorescence intensity ratio obtained from the two threshold values gives Pl(4)P probe amounts in the perinuclear region of the cells. Alternatively, imaging of the Pl(4)P probe was carried out using confocal microscopy. In this case, the Golgi/cytosol Pl(4)P probe fluorescence ratio per cell was determined by dividing the mean fluorescence intensity obtained from three regions of 3 µm² within the Golgi area to the mean fluorescence intensity obtained from three regions of 3 µm² within the cytosol area.

OSBP Proteolysis

Purified OSBP was incubated at 30°C under constant agitation, in the presence of either 1 or 2 µg/ml trypsin. The reaction was stopped at the indicated time by addition of 2 mM PMSF. For activity measurements, the fragments obtained from 250 µg purified OSBP were resolved on a Superose 12 column. Each fraction was then tested for DHE transfer and liposome tethering activity.
DHE and PI(4)P Transfer Assays

All fluorescence experiments were performed in a Shimadzu RF-5301-PC spectrofluorometer using a cylindrical quartz cuvette (600 µL) equilibrated at 37°C and equipped with a magnetic bar for continuous stirring. For most DHE transfer assays using full-length OSBP (Figures 5, 6, and 7), the cuvette initially contained Golgi-like liposomes (Lb) with 2.5 mol % Dansyl-PE (63 µM total lipids) in HK buffer supplemented with 1 mM MgCl2 (HKM buffer). When indicated, VAP-A-His, Arf1-GTP, FFAT peptide, or 25-OH were included in the cuvette. At the indicated times, OSBP (0.1 µM) and ER-like liposomes (La; 63 µM lipid) containing 18 mol % DHE were sequentially added. The sterol transport activity of OSBP was monitored by FRET between DHE and Dansyl, measured at 525 nm (bandwidth: 5 nm) upon excitation at 310 nm (bandwidth: 1.5 nm) (John et al., 2002). Concentrated MCD (1 mM) was used to determine the maximal FRET signal due to full sterol equilibration between Lb and Lc liposomes.

For the DHE transfer experiments of Figures 4A and 6B, liposomes Lc and La displayed the same Golgi-like composition. La also contained 18 mol % DHE and Lc contained 2.5 mol % dansyl-PE.

To analyze the sterol exchange activity of the various Superose 12 fractions, 250 nM VAP-A-His (200-250 nM) and 2 mol % PI(4)P. A first set of 10 autocorrelation curves was acquired to assess the initial size distribution of the liposome suspension. Then tether proteins were added manually (100-250 nM PH-FFAT or 250 nM OSBP), mixed thoroughly and the kinetics of aggregation was followed by acquiring one autocorrelation curve every 10 s (temperature: 30°C). The data were analyzed using the algorithm provided by the Dynamics v6.1 software (Protein Solutions). During the aggregation process, the autocorrelation functions were fitted assuming that the size distribution is a Gaussian function, giving a mean radius and the polydispersity.

Liposome Binding Assay and Liposome Aggregation Measurements

Flotation assays on sucrose gradient were used to determine protein binding to liposomes, as described previously (Bigay et al., 2005). Liposome aggregation induced by OSBP and PH-FFAT was followed by dynamic light scattering (DLS) using a Dynapro apparatus (Protein Solutions) as described (Drin et al., 2008). The sample in HKM buffer initially contained ER-like liposomes (Lc, 25 µM lipids) loaded or not with VAP-A-His (200-250 nM) and Sac1-His (when indicated), and Golgi-like liposomes (Lb, 25 µM lipids) supplemented or not with 200 nM Arf1-GTP and with 2 mol % PI(4)P. A first set of 10 autocorrelation curves was acquired to assess the initial size distribution of the liposome suspension. Then tether proteins were added manually (100-250 nM PH-FFAT or 250 nM OSBP), mixed thoroughly and the kinetics of aggregation was followed by acquiring one autocorrelation curve every 10 s (temperature: 30°C). The data were analyzed using the algorithm provided by the Dynamics v6.1 software (Protein Solutions). During the aggregation process, the autocorrelation functions were fitted assuming that the size distribution is a Gaussian function, giving a mean radius and the polydispersity.

Tethering Assay with Giant Liposomes and Templates

For membrane tethering observation by light microscopy, giant ER-like liposomes were prepared by spontaneous formation as described (Drin et al., 2008). Briefly, a lipid film containing egg PC/brain PS/Rhodamine-PE/DOGS-NiNTA (91, 5, 2, 2 mol %) and 18 mol % DHE (65, 18, 8, 5, 2, 2 mol %) were incubated with 5 x 106 uniform silica beads of 5 µm (Bangs Laboratories) in HK buffer (100 µl total volume) for 30 min at room temperature under regular vortex. Templates were washed three times by centrifugation in HK buffer (200 x g for 2 min). The lipid concentration of the templates was estimated by fluorescence. Tethering was initiated by mixing at room temperature a small volume of giant liposomes loaded with 250 nM VAP-A-His with templates (5 µM lipids) and with 250 nM PH-FFAT or OSBP as indicated. Fluorescence microscopy was carried out using a Confocal Leica TCS SP5 microscope. Images were acquired using a HCX Plan Apo CS 63×/1.4 objective. Alternatively, templates were imaged using wide-field microscopy.

Electron Microscopy

For transmission electron microscopy analysis, cells expressing GFP-VAP-A and mCherry-OSBP or PH-FFAT-mCherry were sorted using a FACSAria III cell sorter (BD Biosciences) and plated for 4 hr. Cells were fixed with 1.6% glutaraldehyde in 0.1 M phosphate buffer, rinsed in 0.1 M cacodylate buffer, and postfixed for 1 hr in 1% osmium tetroxide and 1% potassium ferrocyanide in 0.1 M cacodylate buffer to enhance membrane staining. The cells were then rinsed in distilled water, dehydrated in alcohols and lastly embedded in epoxy resin. Contrast ultrathin sections (70 nm) were analyzed under a JEOL 1400 transmission electron microscope mounted with a Morada Olympus CCD camera. For liposomes negative contrasts, samples were deposited on glow discharge carbon-coated grids and negatively stained with 1% uranyl acetate and then directly observed with the microscope.
Sac1 PI(4)P Hydrolysis Assay

The reactions were carried out in small test tubes in a final volume of 110 µl at room temperature under gentle shaking. Liposomes (500 µM, ER-like) were incubated with 50 nM Sac1-His, with the indicated amount of VAP-A-His and with 200 nM OSBP or 200 nM PH-FFAT. The reaction was initiated by the addition of liposomes (500 µM, egg PC/liver PE/brain PS/PI(4)P: 57/18/5/20 mol%). At the indicated times, an aliquot of the mixture was withdrawn and mixed with 50 mM NEM to stop the reaction. Phosphate concentration was determined by a colorimetric assay (absorbance at 650 nm) using malachite green reagent (Pucadyil and Schmid, 2008).

For the Sac1 cis experiment, ER-like liposomes were supplemented with 20 mol % PI(4)P at the expense of egg PC. In this case, the reaction was initiated by the addition of Sac1.

SUPPLEMENTAL REFERENCES


Figure S1. Characterization of the Membrane Contact Sites Induced by OSBP or by its PH-FFAT Region, Related to Figure 1

(A) HeLa cells expressing mCherry-OSBP (treated or not with 25-OH) or PH-FFAT- mCherry. The localization patterns are similar to that observed in cells co-expressing these constructs with VAP-A-GFP (Figures 1B and 1C).

(B) HeLa cells expressing PH-FFAT-GFP were fixed and immuno-labeled with a TGN46 polyclonal antibody (AbD Serotec) and a donkey anti-sheep AlexaFluor594-conjugated secondary antibody (Invitrogen).

(C and D) Electron micrographs of membrane apposed regions in HeLa cells expressing VAP-A and PH-FFAT in the absence of 25-OH (C) or expressing VAP-A and OSBP and treated with 25-OH (D). The ER is identified by the presence of bound ribosomes (r) and by a dark lumen, whereas the Golgi apparatus (G) is identified by the presence of coated buds (cb) and by a clear lumen.

(E and F) HeLa cells coexpressing GFP-VAP-A and mutants of PH-FFAT-mCherry (E) or mCherry-OSBP (F). When the PH domain cannot interact with PI(4)P (mutation R108L), OSBP and PH-FFAT are found associated with the ER (middle row). When the FFAT motif cannot interact with VAP-A (mutation FF > AA), OSBP and PH-FFAT are found associated with the Golgi (lower row). Thus, OSBP and PH-FFAT use the same membrane determinants to interact with cellular membranes. However, the presence of a functional PH domain and of a functional FFAT motif leads to the formation of extensive ER-Golgi clusters in the case of PH-FFAT, but not in the case of full-length OSBP, which is more soluble than its mutants. Thus, and as summarized in the tables, PH-FFAT follows an additive rule: functional PH + functional FFAT motif = Golgi + ER localization, whereas OSBP follows a different rule: functional PH + functional FFAT motif = cytosol localization. Histograms represent measurements (±SD) performed on 15–20 cells.
Figure S2. OSBP Is Competent for Membrane Tethering In Vitro, Related to Figure 2

(A–C) Experiments similar to that shown in Figure 2 were performed with full-length OSBP (0.2 μM) instead of PH-FFAT and analyzed by DLS (A), EM (B) and light microscopy (C). (C) shows confocal images (top) and 3D reconstructions (bottom) of the same fields to highlight the extended contacts between templates and giant liposomes when OSBP is present.

(D) Dynamic light scattering experiments similar to that shown in (A) were performed in the presence of 25-OH. The plot shows the apparent radius (mean ± SD from 15 autocorrelation curves) at t = 2000 s as a function of 25-OH concentration.
Figure S3. Inhibition of Sterol Biosynthesis at the ER Prevents OSBP from Displacing PI(4)P at the Golgi, Related to Figure 3
(A) Sequence alignment of the ORD of OSBP and of Osh4p and close-up view of the Osh4p-PI(4)P complex (PDB entry: 3SPW). Arrowheads: conserved basic residues involved in PI(4)P interaction and chosen for mutagenesis.
(B) HeLa cells coexpressing GFP-VAP-A and mCherry-OSBP. When sterol biosynthesis is inhibited as in Figure 3C, OSBP and VAP-A concentrate to a perinuclear region.
Figure S4. Gel Filtration Analysis of OSBP or Trypsin-Digested OSBP, Related to Figure 4

(A–C) Elution profiles (as monitored by UV absorbance) of molecular weight standards (A), of full-length OSBP (B), and of trypsin-digested OSBP (C) on a Superose 12 column. The profile shown in (C) corresponds to the experiment shown in Figure 4C. The asterisk indicates the excluded volume of the column.
Figure S5. Mechanism of OSBP Activation, Related to Figure 5

(A) Effect of soluble VAP on OSBP-mediated DHE transfer. The experimental conditions were as Figure 4A (liposomes L_a contained no DOGS-Ni-NTA). VAP-A has a significant stimulatory effect on OSBP-mediated DHE transfer despite the lack of membrane attachment.

(B) Arf1-GTP stimulates OSBP only when bound to liposomes L_b. The experimental conditions were as in Figure 5A except that myristoylated Arf1 was attached to liposomes L_a or L_b, or was replaced by \(\Delta 17\)Arf1-GTP, a soluble form of active Arf1 (Kahn et al., 1992). The error bars indicate the values of two independent experiments.
Figure S6. PI(4)P Inhibits OSBP after a Few Rounds of DHE Transfer, Related to Figure 6

(A) The original recordings of Figure 6A are shown again, together with an expanded view of the first minutes of DHE transfer to highlight the biphasic shape of the kinetics. In this expanded view, the FRET signal is converted into a number (n) of OSBP cycles between liposomes L_a and L_b. This number equals \( n = \frac{(F/F_0 - 1)}{(F_{\text{max}}/F_0 - 1) / 0.09 / 0.1} \), where F is the fluorescence level at time t, F_0 is the initial fluorescence level, F_{\text{max}} is the final fluorescence level after DHE equilibration between liposomes L_a and L_b (as determined by the addition of MCD, horizontal dashed line), and \( 0.09 / 0.1 \) is the total number of cycles that OSBP has to undergo in order to equilibrate DHE between L_a and L_b. Indeed, the concentration of OSBP is 0.1 \( \mu \text{M} \) and the concentration of DHE that equilibrates between L_a and L_b equals 5.7 \( \mu \text{M} \) (\( = 63 \times 0.18 / 2 \) since L_a and L_b were used at 63 \( \mu \text{M} \) and contained 0 and 18 mol % DHE, respectively). This calculation leads to 57 cycles. Overall, PI(4)P clearly inhibits DHE transfer in a dose-dependent manner. However, this inhibitory effect applies only after the first fifty seconds of DHE transfer. Initially, the time courses with or without PI(4)P are roughly parallel, suggesting that OSBP is not inhibited by PI(4)P during the first cycle of DHE transfer.

(B) Quantification of the data shown in A as determined from linear fits of the second phase.
The four-step cycle of OSBP is inhibited by 25-OH. The experimental conditions were similar to that used in Figure 6A and 7E with the following modifications. Transfer of DHE between liposomes L_a (63 μM lipids + 1 μM VAP-A) and L_b (63 μM lipids, 2 mol % PI(4)P) was measured in the presence of 0.1 μM OSBP. When indicated, 50 nM Sac1 and/or 10 μM 25-OH were added. 25-OH inhibits DHE transfer under these turnover conditions where OSBP exchanges DHE for PI(4)P, which is then hydrolyzed by Sac1. Dashed line: maximal fluorescence observed after DHE equilibration with 1 mM MCD.

Coiled-coil probability in the N-ter sequence of OSBP using a sliding window of 21 aa as determined by COILS (http://www.ch.embnet.org/software/COILS_form.html) (Lupas et al., 1991). Two coiled-coil regions of 30 and 50 aa, respectively, are predicted, although only the first one was identified in early studies on OSBP (Dawson et al., 1989).

Putative domain organization of OSBP at the interface between two membranes. The aim of this model is to show protein domains at scale, thus allowing comparison with the distance between apposed membranes as observed experimentally (~20 nm; see Figure 1D). This model was constructed using the structures of the PH domain of ORP11 (2D9X), the ORD domain of Osh4p (1ZH2), the soluble region of VAP-A in complex with a FFAT motif (1Z9O), and Arf1-GTP (1O3Y). Coiled-coil regions were modeled according to the structure of tropomyosin (2D3E). This model also suggests questions for the functioning of OSBP at membrane interfaces, including the roles of protein dimerization and of linker regions. Notably, the linker between the FFAT motif and the ORD domain contains ~50 amino acids, which corresponds, in the case of a random amino acid chain, to an end-to-end distance of (130 × 50)^1/2 = 80 Å (Creighton, 1993). This length seems barely enough to allow the ORD domain flipping between apposed membranes, thus leaving the possibility that, at some point of the lipid exchange cycle, the FFAT motif dissociates from VAP-A. It is interesting to compare this model, in which the membrane are closely apposed, to another model derived from the structural analysis of the individual domains of the OSBP homolog Osh3p. In this case, the distance between the two membranes is proposed to be much larger because the protein does not seem to contain coiled-coil regions but rather extended and unstructured regions (Tong et al., 2013).