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The Diverse Functions of Oxysterol-Binding Proteins

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Abstract

Oxysterol-binding protein (OSBP)-related proteins (ORPs) are lipid-binding proteins that are conserved from yeast to humans. They are implicated in many cellular processes including signaling, vesicular trafficking, lipid metabolism, and nonvesicular sterol transport. All ORPs contain an OSBP-related domain (ORD) that has a hydrophobic pocket that binds a single sterol. ORDs also contain additional membrane binding surfaces, some of which bind phosphoinositides and may regulate sterol binding. Studies in yeast suggest that ORPs function as sterol transporters, perhaps in regions where organelle membranes are closely apposed. Yeast ORPs also participate in vesicular trafficking, although their role is unclear. In mammalian cells, some ORPs function as sterol sensors that regulate the assembly of protein complexes in response to changes in cholesterol levels. This review will summarize recent advances in our understanding of how ORPs bind lipids and membranes and how they function in diverse cellular processes.

Keywords

cholesterol; sterol; phosphoinositides; signaling; lipid transport; membranes; membrane contact sites; lipid transport proteins

INTRODUCTION

Intracellular membranes in eukaryotic cells have specific lipid compositions that are required for them to function properly. How the lipid composition of membranes is sensed and regulated remains, in most cases, poorly understood. One group of proteins that plays an important role in these processes is known as lipid transfer proteins (LTPs). These proteins were initially discovered, as their name indicates, because of their ability to transfer lipids between biological membranes or liposomes in vitro. However, in most cases it remains unclear if these proteins actually transfer lipids in cells or if they sense and regulate the lipid composition of membranes by other mechanisms.

After the discovery and sequencing of the first LTPs, it became clear that most species contain numerous LTPs and that they fall into several families on the basis of sequence similarity. The structure of at least one member of each of these families has been solved and, although they are structurally distinct, LTPs share a few common features. They all contain a core lipid-binding domain that has a hydrophobic pocket that can accommodate a single lipid molecule. In many cases, a mobile “lid” domain covers the pocket, shielding the

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bound lipid from the aqueous environment. In addition to this core lipid-binding domain, many LTPs contain other motifs and domains. These features suggest that LTPs could perform at least one of three main functions in cells. First, they could transfer lipid monomers between membranes by extracting a lipid from one membrane and delivering it to a second membrane. Second, they could act as sensors that might, for example, alter their interaction with other proteins in response to binding or releasing a lipid. Finally, LTPs could act as “lipid presenters” that offer lipids to metabolic enzymes. Such a role has been proposed for Sec14p, a yeast protein that is a member of the phosphatidylinositol (PI)/phosphatidylcholine (PC) transfer protein family. This protein binds PI and presents it to some PI kinases (Schaaf et al. 2008).

The oxysterol-binding protein (OSBP)-related proteins (ORPs) are a large family of LTPs that are conserved from yeast to humans. They have been implicated in many cellular processes including signaling, vesicular trafficking, lipid metabolism, and nonvesicular sterol transfer. This review summarizes what is known about how these proteins interact with lipids and membranes and how they might function in cells.

IDENTIFICATION OF OSBP AND ORPs

Oxysterols are derivatives of cholesterol that can be produced by both enzymatic and nonenzymatic reactions, although they are usually found only at very low levels in cells (Bjorkhem & Diczfalussy 2002, Russell 2000). Early studies on feedback inhibition of cholesterol biosynthesis by sterol indicated that oxysterol was 50 times more potent than cholesterol itself (Brown & Goldstein 1974, Kandutsch et al. 1978). These and other findings prompted a search for an oxysterol-binding protein that led to the identification of OSBP (Taylor et al. 1984). The protein was later purified and the cDNAs from rabbits and humans cloned (Dawson et al. 1989a,b; Levanon et al. 1990).

The cloning of OSBP eventually led to the identification of several homologs (the ORPs) from various eukaryotes. All contain the ORP signature motif EQVSHHPP and the core lipid binding ORP domain (ORD). Most also contain additional motifs and domains. The most-studied ORPs are those of humans and the yeast *Saccharomyces cerevisiae*. Humans have 12 ORP genes, and splicing generates 16 different protein products (Lehto et al. 2001). Yeast has 7 ORP genes (Beh et al. 2001) called *OSH1-7*, although some have additional names as well.

Most ORPs in humans, and some in yeast, contain other domains in addition to the lipid-binding ORD. The domain structure of human and yeast ORPs is shown in Figure 1. Many ORPs contain two phenylalanines in an acidic tract (FFAT) motif and pleckstrin homology (PH) domains. FFAT motifs are found in many proteins involved in lipid metabolism and are bound by endoplasmic reticulum (ER)-resident proteins called vesicle-associated membrane protein-associated proteins (VAPs) in humans and Scs2p in yeast (Loewen et al. 2003). Many PH domains bind phosphoinositides (PIPs) although many bind PIPs with only low affinity and likely have other ligands as well (Lemmon 2007). Some ORPs also contain ankyrin repeats and Golgi dynamics (GOLD) domains, which are thought to mediate protein-protein interactions, but their role in ORPs is not known. Two of the human ORPs also contain single C-terminal transmembrane domains.

ORPs have also been identified in several other species. For example, the mouse genome contains 12 ORP genes with a high degree of homology to human ORPs. They produce 12 proteins with distinct tissue distributions and are all expressed in early embryos, indicating a possible role in embryogenesis (Annis et al. 2002). We have constructed a phylogenetic tree of all of the known and some putative ORP sequences from a variety of organisms (Figure 2). It reveals that ORPs are a complex family of proteins and that multiple copies of

the genes encoding ORPs probably existed in the common ancestor of plants, fungi, and animals. Interestingly, one group of ORPs is fungus-specific. Whether this group performs a function or binds a lipid that is unique to fungi is not known.

STRUCTURE OF Osh4p/Kes1p

The yeast ORP Osh4p, also known as Kes1p, is the only ORP with a known structure (Im et al. 2005). This ORP has 435 amino acids and contains the core ORD domain found in all ORPs but lacks the other domains present in many ORPs, such as a PH domain or FFAT motif (Figure 1). The structure of Osh4p/Kes1p was solved with the protein bound to cholesterol, to one of several oxysterols, or to ergosterol, the primary sterol in yeast (Figure 3*a*). All these structures were virtually identical and revealed that the protein is built around a 19-strand antiparallel β -sheet that forms a nearly complete β -barrel, a fold that is unique among LTPs. Remarkably, the barrel is similar to that of transporters found in the outer membranes of bacteria and mitochondria. The bound sterols were all similarly positioned within the central tunnel of the β -barrel, which contains many hydrophobic residues. Surprisingly, there were no direct interactions between the hydroxyl groups in the oxysterols (other than the 3-hydroxyl group common to them all) and the protein, suggesting that the protein can bind a wide range of sterols with similar specificity. The importance of the central tunnel for cholesterol binding and the ability of the protein to function in cells was confirmed by mutational analysis (Im et al. 2005, Raychaudhuri et al. 2006). A lid domain that shields the bound sterol from the aqueous phase covers the sterol-binding tunnel. The 3-hydroxyl groups of the sterols are located at the bottom of the tunnel whereas the side chains make contacts with the hydrophobic inner surface of the lid. Other LTPs also bind their ligands in hydrophobic pockets or tunnels, although with different structures (Haapalainen et al. 2001, Roderick et al. 2002, Schaaf et al. 2008, Tsujishita & Hurley 2000).

The structure of Osh4p/Kes1p was also solved in absence of ligand (Figure 3*b*). Although the full-length protein did not crystallize without ligand, crystals were obtained from a protein lacking the N-terminal lid domain and the surface loop 236-240 (a portion of Loop A in Figure 3*a*) (Im et al. 2005). It is likely that, in the absence of ligand, both these regions of the protein are highly mobile. In the apo state, the tunnel is open and helix $\alpha 7$ (Figure 3) pivots about 15 Å so that a relatively flat surface is exposed that could bind membranes during sterol extraction from or delivery to membranes.

LIPID BINDING BY ORPs

Sterol Binding

OSBP was originally isolated because of its ability to bind oxysterols. It has subsequently been found that it also binds cholesterol (Wang et al. 2005c). OSBP binds cholesterol and 25-hydroxycholesterol (25-OH-cholesterol) with K_d values of approximately 70 nM and 5 nM, respectively (Wang et al. 2008). The yeast ORP Osh4p/Kes1p binds these sterols with similar affinities (Im et al. 2005). It also binds ergosterol and many other oxysterols. Less is known about the ability of other ORPs to bind sterols. Using photo-crosslinkable derivatives of cholesterol and 25-OH-cholesterol, Olkkonen and coworkers found that most human ORPs bind either compound (Suchanek et al. 2007). Similarly, our group has found that all yeast ORPs have the ability to transfer sterols between liposomes in vitro, although some do so only very poorly (Schulz et al. 2009). This suggests that most yeast ORPs can bind sterols. It remains possible, however, that some ORPs primarily bind lipids other than sterols. The structure of Osh4p/Kes1p revealed that the protein makes relatively few contacts with bound sterol and that residues in the binding tunnel are, for the most part, not well conserved among ORPs (Im et al. 2005).

ORPs probably bind and release sterols by extracting them from or delivering them to membranes. This process has been most carefully studied for Osh4p/Kes1p, which can rapidly extract and deliver cholesterol to liposomes in vitro (Raychaudhuri et al. 2006). Sterols can only enter and exit the binding pocket of Osh4p/Kes1p when the lid is open and away from the entrance of the binding tunnel. When the lid is open, the protein exposes a relatively flattened surface that may interact with a bilayer, allowing the protein to extract or deliver sterols to the membranes (Im et al. 2005). Many conserved basic residues surround the mouth of the sterol-binding pocket, and mutation of these dramatically reduces the ability of the protein to extract sterols from membranes (Im et al. 2005). Molecular dynamics simulations were used to study the movement of sterols out of the binding pocket. These suggest that the rate-limiting step is opening the lid, which is energetically unfavorable in aqueous solution (Canagarajah et al. 2008, Singh et al. 2009). Lid opening may be triggered by its interaction with membranes. Recently, Antonny and coworkers found that the lid of Osh4p/Kes1p forms a membrane-binding α -helix called an ArfGAP1 lipid packing sensor (ALPS) motif (Drin et al. 2007). This motif is found in many proteins, though not most ORPs, and binds preferentially to membranes with high positive curvature. Binding of the ALPS motif in the lid to a membrane might open it and allow sterol to enter or exit the binding tunnel.

Although many ORPs do not seem to contain an ALPS motif, membrane binding may be a common feature of their lid domains. Interestingly, recent evidence suggests that the lid domain of OSBP (which does not have an ALPS motif) has a sterol-binding site. Fragments of OSBP that were missing most of the ORD domain that is homologous to Osh4p/Kes1p were found to retain the ability to bind sterols (Wang et al. 2008). All fragments that retained sterol binding contained the OSBP region that corresponds to the N-terminal lid in Osh4p/Kes1p. Indeed, the authors suggest that in OSBP the lid domain, rather than the sterol-binding tunnel in the β -barrel, is the primary sterol-binding domain. They propose that a sterol initially binds the lid domain, which subsequently moves the sterol into the binding tunnel. A Gly-Ala enriched domain near the N terminus of OSBP also appears to regulate cholesterol binding through an unknown mechanism.

The lid domain of OSBP may have affinity not for free sterols but for sterols in membranes or detergent micelles (Wang et al. 2008). It might, therefore, be similar to the ALPS domain in the lid of Osh4p/Kes1p. In contrast to OSBP, the lid domain of Osh4p/Kes1p does not appear to play an essential role in sterol binding by this protein. A mutant lacking this lid domain retains the ability to bind sterols, although with a decreased affinity, and many mutations outside of the lid domain dramatically reduce or eliminate cholesterol binding without altering the secondary structure of the protein (Im et al. 2005). Therefore, at least for Osh4p/Kes1p, the primary site of sterol binding is in the tunnel formed by the β -barrel and not the lid domain as in OSBP.

PIP Binding

Most ORPs have a PH domain that is N-terminal to the ORD. These domains often bind PIPs (Lemmon 2007). Because different PIP species are enriched on various intracellular membranes, binding of specific PIPs by PH domains can promote localization of ORPs on specific cellular compartments. Many of the PH domains in ORPs have been shown to bind PIPs and contribute to ORP localization in cells (Fairn & McMaster 2005b; Johansson et al. 2005; Lehto et al. 2004; Levine & Munro 1998, 2001; Roy & Levine 2004; Wyles & Ridgway 2004; Xu et al. 2001).

The role of PH domains in the targeting of ORPs has been most extensively studied for OSBP. The localization of OSBP to the trans-Golgi network (TGN) is mediated by its PH domain, which binds phosphoinositide 4-phosphate [PI(4)P] (Levine & Munro 1998, Levine

& Munro 2002). Addition of 25-OHcholesterol to cells causes the protein to become more enriched on the TGN, perhaps because the protein undergoes a conformational change that unmask the PH domain (Ridgway et al. 1992). The TGN localization of the PH domain from OSBP also depends on the small GTPase ADP-ribosylation factor (ARF), which plays a role in transport vesicle formation (Levine & Munro 2002). Interestingly, a direct interaction of the PH domain and ARF has been demonstrated (Godi et al. 2004). Thus, the PH domain may serve as a coincidence detector that drives the localization of OSBP to TGN membranes where both PI(4)P and ARF are located.

Some ORDs can also bind PIPs. Two of the four yeast ORPs that lack PH domains, Osh4p/Kes1p and Osh6p, have been shown to bind PIPs (Li et al. 2002, Wang et al. 2005a). To study PIP binding by Osh4p/Kes1p, Bankaitis and coworkers used a photo-crosslinkable derivative of phosphatidylinositol 4,5-bisphosphate [PI(4,5)P₂] to show that this lipid is bound by the protein with a *K_d* of approximately 2.5 μM. PI(4,5)P₂ and PI(4)P were competing ligands but other PIPs were not, suggesting that binding is specific for these PIPs. PIP binding is probably a common feature of ORDs, but this has not yet been tested. Like the PH domain in OSBP and other ORPs, PIP binding by ORDs probably helps mediate their localization; the enrichment of Osh4p/Kes1p on the Golgi complex requires PI(4)P-binding (Li et al. 2002).

Mutations that ablate PIP binding by Osh4p/Kes1p have been identified, and they are located in a loop of the protein that is distal from the mouth of the sterol-binding pocket (Loop A, Figure 3a) (Li et al. 2002). This suggests that PIPs bind the surface of the protein rather than in the binding pocket. This location is surprising because it has been found that PI(4,5)P₂ stimulates cholesterol transfer by Osh4p/Kes1p between liposomes (Raychaudhuri et al. 2006). How this stimulation occurs is not clear but presumably requires that the protein interact with PI(4,5)P₂ while it is extracting or delivering sterol to a membrane. However, it is difficult to see how this occurs if movement of a sterol into or out of the pocket requires that the mouth of the pocket make close contacts with the membrane. It is possible that the protein undergoes a conformational change when it binds a PIP that allows it to simultaneously extract or deliver a sterol. Alternatively, it could bind a single negatively curved membrane or two membranes simultaneously. We have recently found that Osh4p/Kes1p and all the ORDs in yeast have the ability to bind two membranes simultaneously (Schulz et al. 2009).

Although Osh4p/Kes1p binds PIPs at a site on the surface of the protein, outside the sterol-binding pocket, evidence suggests that PI(4,5)P₂ can bind in the β-barrel pocket as well. Osh4p/Kes1p can extract PI(4,5)P₂ from liposomes and transfer it to a second set of liposomes, albeit rather poorly (Raychaudhuri et al. 2006). It seems unlikely that the protein could extract and transfer PI(4,5)P₂ when the lipid is bound to the surface of the protein; PI(4,5)P₂ must therefore be able to enter the binding tunnel. Because the charged head-group of PI(4,5)P₂ probably cannot be accommodated in the hydrophobic binding tunnel, it seems more likely that its acyl chains bind there.

OTHER DOMAINS COMMONLY FOUND IN ORPs

FFAT Motif

VAPs are ER-resident integral membrane proteins (Skehel et al. 1995) that bind proteins containing FFAT motifs, many of which are cytosolic lipid-binding proteins (Loewen et al. 2003). Most mammalian ORPs and three of the seven ORPs in yeast contain this motif. The interaction between VAPs and proteins with FFAT motifs is thought to target these proteins to the ER. For the mammalian ORPs, this has been demonstrated for OSBP, ORP3, ORP6, ORP7, and ORP9 (Lehto et al. 2004, Lehto et al. 2005, Wyles & Ridgway 2004, Wyles et al.

2002). In *S. cerevisiae*, Scs2p is the primary VAP homolog (Loewen & Levine 2005). It binds several FFAT-containing proteins in yeast, including the ORPs Osh1p, Osh2p, and Osh3p (Gavin et al. 2002, Loewen et al. 2003).

Ankyrin Repeat Domains

Some ORPs, including the mammalian ORP1L and *S. cerevisiae* Osh1p and Osh2p, have N-terminal ankyrin repeat motifs, which are known to mediate protein-protein interactions in a wide variety of other proteins (Li et al. 2006). The ankyrin domain in Osh1p probably interacts with the protein Nvj1p, which is required for the localization of Osh1p to a subdomain of the ER known as the nucleus-vacuole junction (NVJ) (Kvam & Goldfarb 2004, Levine & Munro 2001). This is a region of close apposition of these two organelles. Such zones of close contact, typically between the ER and a second organelle, have been called membrane contact sites (MCSs) and will be discussed below. The ankyrin repeat in ORP1L also contributes to the localization of this protein to a MCS, in this case between the ER and late endosomes. The ankyrin repeat region of ORP1L binds the late endosome small GTPase Rab7 (Johansson et al. 2005). When cellular cholesterol is low, ORP1L undergoes a conformational change that allows its ankyrin repeat region to bind Rab7 while it simultaneously binds VAP in the ER (via its FFAT motif), which induces the formation of close contacts between the endosomes and the ER (Rocha et al. 2009).

MULTIPLE MEMBRANE BINDING SURFACES ON ORDs

Recent work from our laboratory has suggested that the ORDs from all seven ORPs in *S. cerevisiae* have more than one membrane-binding surface (Schulz et al. 2009). We found that all these ORDs had the ability to cause liposome aggregation, probably as monomers, suggesting that they have multiple membrane-binding surfaces. The membrane-binding surfaces of Osh4p/Kes1p were examined in more detail and, consistent with other work on this protein, Osh4p/Kes1p was found to have at least four (Figure 3). One is near the surface of the mouth of the sterol binding tunnel (Im et al. 2005). A second is in the lid domain (Drin et al. 2007). At least two other membrane-binding sites are distal from these regions, and both have been implicated in PIP binding (Loops A and B, Figure 3a) (Li et al. 2002, Schulz et al. 2009). Ablation of one of these two binding surfaces (Loop B, Figure 3a) eliminates PI(4,5)P₂-dependent regulation of sterol transport by Osh4p/Kes1p. This mutant protein is also not functional in cells (Schulz et al. 2009). It seems likely that most ORDs in higher eukaryotes, like those in yeast, have multiple membrane-binding surfaces as well. Indeed it has been found that the ORD from ORP1S can bind both sterols and PIPs (Fair & McMaster 2005a, Suchanek et al. 2007), suggesting that it also has more than one membrane-binding surface. Understanding how the multiple membrane-binding surfaces on ORPs regulate lipid binding by these proteins will be important for unraveling how they function in cells.

Little is known about the affinity of most ORPs for membranes or how their membrane association is regulated. Interestingly, Vps4p, an ATPase in the AAA (ATPases associated with a variety of cellular activities) family, may regulate the membrane association of the yeast ORPs Osh6p and Osh7p (Wang et al. 2005b). Vps4p plays a critical role in multivesicular body formation, where it facilitates the dissociation of large protein complexes from the membrane (Davies et al. 2009). Vps4p directly binds to Osh6p and Osh7p, and the fraction of Osh6p and Osh7p that is membrane associated increased substantially in cells and cell extracts missing Vps4p. Yang and coworkers suggest that Vps4p could use ATP to drive membrane dissociation of Osh6p and Osh7p (Wang et al. 2005b, Yang 2006). However, this has not yet been directly demonstrated.

LOCALIZATION AND MEMBRANE CONTACT SITES

Most ORPs lack transmembrane domains, with the exception of ORP5 and ORP8, and are cytosolic proteins. They also can be targeted to two different organelles: to the ER, via the FFAT motif, and to organelles enriched in the PIPs recognized by their PH domains. The multiple targeting domains found in most ORPs may allow them to be enriched on two different organelles or to shuttle between them. However, the multiple domains could also promote ORP localization at MCSs, where the ER and a second organelle are closely apposed. It has been proposed that ORPs (and other LTPs) might operate primarily at MCSs (Holthuis & Levine 2005, Levine & Loewen 2006). At MCSs, ORPs might be able to touch both membranes simultaneously or to shuttle rapidly between two organelles.

Many ORPs in yeast are enriched at MCSs. Osh1p localizes to the NVJ, where the nucleus and the vacuole are closely apposed (Levine & Munro 2001). This MCS requires the interaction of two proteins, Nvj1p in the ER and Vac8p on the vacuolar membrane. Nvj1p is an integral membrane protein (Pan et al. 2000); Vac8p is soluble but probably requires palmitoylation to be localized to the NVJ (Peng et al. 2006). The functions of the NVJ and of Osh1p at the NVJ remain unknown. The NVJ could be a site of lipid transfer between the ER and vacuole, some of which could be mediated by Osh1p (Kvam & Goldfarb 2004). It is also the site of piecemeal microautophagy of the nucleus, which results in the pinching-off of portions of the nucleus into the vacuole (Krick et al. 2008, Roberts et al. 2003). This process is not disrupted in cells missing Osh1p but is in a mutant lacking all yeast ORPs (Kvam & Goldfarb 2004).

Other yeast ORPs may be located at MCSs between the ER and the plasma membrane (PM). Osh2p, Osh3p, and Osh6p are enriched in patches at the cell cortex (adjacent to the PM) that have been suggested as ER-PM contact sites (Loewen et al. 2003, Wang et al. 2005a,b). We have also found a similar localization for Osh7p (Schulz et al. 2009). Osh3p is not normally localized at the cell cortex but rather to puncta that are probably Golgi complexes; Scs2p overexpression caused Osh3p to relocate to the cortex (Loewen et al. 2003). Similarly, Osh2p was partially localized to the cortex in wild-type cells but, like Osh3p, its localization at the cortex was enhanced by Scs2p overexpression. Mutation of the FFAT motif in Osh2p and Osh3p caused them to be cytosolic. Consistent with these findings, we have found that Osh proteins at the cortex colocalize with the ER, suggesting that they may be enriched at sites where the ER and PM are closely apposed (Schulz et al. 2009). Osh proteins at PM-ER contact sites could transfer sterols or other lipids between these organelles, or they might regulate other proteins at these sites, such as the PIP kinase Stt4p complex (Baird et al. 2008). The fact that even the core ORD domain found in all ORPs can interact with two membranes simultaneously (Schulz et al. 2009) suggests that ORPs are well suited to sense and integrate signals from two organelles at contact sites. Understanding the localization of yeast ORPs (and other proteins) at PM-ER contact sites will require a better characterization of MCSs with electron microscopy.

Some mammalian ORPs may be located at MCSs as well. ORP1L plays a role in regulating close contacts between late endosomes and the ER. This ORP localizes to late endosomes, where it forms a complex with the GTP-bound form of the small GTPase Rab7 and a protein called RILP (Rab7 interacting lysosomal protein) (Johansson et al. 2005). In a beautiful set of experiments, Neefjes and coworkers showed that this complex mediates the interaction of late endosomes with either a motor protein complex or the ER, depending on the cholesterol concentration in cells (Rocha et al. 2009). When cholesterol levels are high, RILP (which is in complex with ORP1L and Rab7) interacts with the p150^{Glued} subunit of the dynein-dynactin motor (Johansson et al. 2007). A decreasing cellular cholesterol level causes ORP1L to undergo a conformational change and bind to VAP in the ER, bringing the ER

and late endosomes into close contact and displacing p150^{Gluc}. The authors propose that ORPIL is acting as a cholesterol sensor that causes late endosomes and the ER to associate when cholesterol levels are low. Whether it also facilitates cholesterol transfer between these organelles in these conditions remains a question.

There is some evidence to suggest that OSBP and ORP9 may also localize to MCSs, specifically those between the ER and the Golgi complex. Both proteins have been implicated in Golgi complex function and could operate at ER-Golgi complex contact sites, either to regulate lipid transfer by other LTPs or to directly move cholesterol between these organelles (Ngo & Ridgway 2009, Peretti et al. 2008).

FUNCTIONS OF ORPs

ORPs have been implicated in a variety of cellular processes including cell signaling, vesicular trafficking, lipid metabolism, and nonvesicular lipid transport. Given what is known about how ORPs bind lipids and membranes and where they are localized in cells, they might use at least four types of mechanisms (Figure 4). First, they could move lipids between cellular membranes through cycles of lipid extraction and delivery (Figure 4*a*). This probably occurs largely at MCSs. Second, ORPs might help establish transient changes in the distribution of lipids within a membrane by adding or removing lipids from a region of the membrane (Figure 4*b*). In this way ORPs could contribute to processes such as membrane bending or signaling that can be driven by temporary clustering or exclusion of particular lipids in regions of a membrane (Balla et al. 2009, Bard & Malhotra 2006, Chernomordik & Kozlov 2003, De Matteis & Godi 2004). Third, they could function as lipid sensors that alter their interactions with other protein partners in response to binding or releasing lipid ligands. Lipid sensing could be harnessed to a signaling event or to regulating the interactions of proteins or membranes. Lipid sensing by ORPs may occur primarily at organelle contact sites (Figure 4*c*). Finally, ORPs could regulate the access of other lipid-binding proteins to the membrane, either by presenting a lipid to a second lipid-binding protein (Figure 4*d*) or by preventing the lipid-binding protein from accessing a lipid in the membrane (Figure 4*e*). These mechanisms are not mutually exclusive, and some ORPs could use more than one of them. In the following sections, some functions of ORPs in *S. cerevisiae* and in mammals are discussed.

ORPs in Yeast

The ORPs in *S. cerevisiae* have a single overlapping essential function because any one of the seven ORPs in yeast is required for viability (Beh et al. 2001). None of the seven genes encoding ORPs in yeast, called Osh proteins, are essential for viability. However, eliminating all Osh proteins is lethal. A strain was created in which all seven *OSH* genes were deleted but a plasmid with a temperature-sensitive allele of *OSH4* (*osh4Δ osh4-1*) was added. When this strain is shifted to the nonpermissive temperature, it accumulates transport vesicles and has defects in endocytosis and maintenance of vacuole integrity (Beh & Rine 2004, Beh et al. 2001). It also has an altered intracellular distribution of sterols. This was assessed with filipin, a dye that fluoresces when it complexes with free sterols. In wild-type cells most free sterol is in the PM (Zinser et al. 1991), and thus filipin fluorescence is almost entirely in the PM (Beh & Rine 2004). When *osh4Δ osh4-1* cells are shifted to the nonpermissive temperature, there is a substantial amount of filipin fluorescence in the internal compartment and a decrease occurs in the PM, indicating that intracellular sterol distribution is perturbed.

The single shared essential function of ORPs in yeast is not known. It is important, however, that Osh4p/Kes1p is by far the most abundant of the ORPs in yeast. It has been estimated that there are approximately 32,000 copies of this Osh4p/Kes1p per cell whereas the

abundance of the other six ORPs ranges from 850 to 2500 copies per cell (Fairn & McMaster 2008). Therefore, the single essential function that all the Osh proteins can perform may only require a small number of copies of an Osh protein. However, it is possible that in cells lacking six of the seven Osh proteins, the expression of the remaining Osh protein substantially increases, but this has not been determined.

Work to date has suggested two possible common functions that all Osh proteins in yeast may share: sterol transfer between cellular membranes and Rho-dependent polarized vesicular transport.

ORPs and nonvesicular sterol transport—In yeast, sterol transfer between the ER and PM does not require many of the Sec proteins needed for vesicular trafficking, which suggests that nonvesicular sterol transport occurs between these organelles (Baumann et al. 2005, Li & Prinz 2004, Schnabl et al. 2005). Sterol transfer between the ER and PM was studied in *osh4Δ osh4-1* cells. At the nonpermissive temperature, the movement of exogenous sterol from the PM to the ER slows substantially (Raychaudhuri et al. 2006). ER to PM sterol transfer of newly synthesized sterol is also dramatically reduced in these cells (Beh et al. 2009). These findings suggest that, collectively, the ORPs in yeast are required for nonvesicular sterol transfer between the ER and PM. Several ORPs must participate because mutants missing just one of these proteins had only modest decreases in PM to ER sterol transfer, although this analysis suggested that Osh3p and Osh5p play an important role (Raychaudhuri et al. 2006). Consistent with a direct role for yeast ORPs in sterol transfer, Osh4p/Kes1p can transfer cholesterol between liposomes in vitro. Subsequently, it was shown that the ORDs from all yeast Osh proteins were able to transfer cholesterol in vitro, although some did so only very poorly (Schulz et al. 2009). Whether some ORPs transfer sterols in mammalian cells remains unknown. Recent work demonstrated that OSBP and ORP9L can transfer cholesterol in vitro and suggests that ORP9L may transfer cholesterol in cells as well (Ngo & Ridgway 2009).

Interestingly, PIPs may regulate sterol transfer by ORPs in yeast. Sterol extraction and transfer by Osh4p are stimulated by PI(4,5)P₂ in vitro. In addition, PM to ER cholesterol transfer slows in cells depleted of PIPs (Raychaudhuri et al. 2006). This suggests that one function of PIP binding by ORPs is to regulate sterol transfer by these proteins. Cholesterol transfer by OSBP and ORP9L was also regulated by PIPs, in this case PI(4)P (Ngo & Ridgway 2009). Whether PIPs regulate sterol binding by most ORPs remains to be determined.

ORPs and polarized growth in yeast—The growth of the budding yeast *S. cerevisiae* requires polarized vesicular trafficking from the mother to the daughter cell. Yeast ORPs are required for this process, though their role is not yet understood (Kozminski et al. 2006). Polarized growth requires the transfer and fusion of transport vesicles to the sites in the daughter cells where localized membrane growth occurs. In cells lacking Osh proteins (*osh4Δ osh4-1* cells) these transport vesicles accumulate in the cell. Polarized secretion in yeast requires two Rho family small GTPases, Cdc42p and Rho1p. These are normally localized at the tip of the growing daughter cells but are not in *osh4Δ osh4-1* cells. Expression of most Osh proteins restores localization and rescues conditional *cdc42* mutants (Kozminski et al. 2006). The Osh proteins were also necessary for the proper localization of Sec4p, another small GTPase that is required for polarized vesicular trafficking. Interestingly, GFP-Sec4p accumulates in the daughter cell, probably on vesicles that have moved to the daughter but fail to dock. How Osh proteins affect polarized vesicular trafficking and whether they directly regulate Rho1p, Cdc42p, or Sec4p remain to be determined. Another important question is whether the ability of Osh proteins to bind and transfer sterol plays a role in these processes.

Role of Osh4p/Kes1p in TGN vesicular trafficking—There are many lines of evidence that Osh4p/Kes1p plays a role in vesicular trafficking at the TGN. The first came from studies on Sec14p, a PI/PC transfer protein involved in vesicle biogenesis. The *SEC14* gene is essential. However, it is possible to isolate “bypass” suppressors of conditional *SEC14* alleles that allow cells with these alleles to grow at the nonpermissive temperature. Some of these suppressors have defects in the gene encoding Osh4p/Kes1p (Fang et al. 1996). Remarkably, deletion of *OSH4* bypasses conditional defects in two other proteins needed for vesicular trafficking at the TGN: Pik1p and Drs2p (Li et al. 2002, Muthusamy et al. 2009). The PI 4-kinase Pik1p is primarily localized at the Golgi complex, where it generates a critical pool of PI(4)P needed for vesicular trafficking (Hama et al. 1999, Strahl et al. 2005, Walch-Solimena & Novick 1999). Drs2p is a type IV P-type ATPase phospholipid translocase needed for vesicle formation in the TGN (Graham 2004). The finding that eliminating Osh4p/Kes1p can bypass the requirement for many proteins that modulate the lipid composition or distribution in the TGN suggests that Osh4p/Kes1p also plays a critical role in these processes. It also suggests that Osh4p/Kes1p may function as an inhibitor of transport vesicle formation in the TGN because removing Osh4p/Kes1p allows cells with defects in this process to survive.

A positive role for Osh4p/Kes1p in vesicular trafficking is suggested by the finding that cells lacking this protein have a defect in delivering some GFP-tagged proteins to the PM (Proszynski et al. 2005). This finding is consistent with other genetic evidence from McMaster and coworkers. Using an elegant genetic screen they found that Osh4p/Kes1p is needed for the function of the Rab GTPase Ypt31p and the transport protein particle II (TRAPP II) complex (Fairn et al. 2007), which are required for some vesicular trafficking in the TGN (Benli et al. 1996, Morozova et al. 2006).

The role of Osh4p/Kes1p in vesicle trafficking is not known. Whatever role it plays in Golgi complex function, however, the other yeast ORPs cannot substitute for it; even when the other Osh proteins are produced at levels comparable with Osh4p/Kes1p, they do not complement a *sec14^{ts} osh4Δ* strain (Fang et al. 1996, Li et al. 2002). This is particularly surprising in the case of Osh5p, which is 69% identical to Osh4p/Kes1p, and suggests that Osh4p/Kes1p has a unique property not shared with other yeast ORPs. Interestingly, mammalian ORP1S and ORP9S can substitute for Osh4p/Kes1p (Fairn & McMaster 2005b, Xu et al. 2001); thus the function of Osh4p/Kes1p is shared by at least two other ORPs, just not ORPs in yeast.

Two models for the function of Osh4p/Kes1p in Golgi complex vesicular trafficking have been proposed. Bankaitis and coworkers recently demonstrated that Sec14p presents PI to the kinase Pik1p, which may allow it to generate a critical pool of PI(4)P in the TGN that is needed for vesicle formation (Schaaf et al. 2008). They propose that Osh4p/Kes1p inhibits Pik1p and that this kinase can produce more PI(4)P in Golgi membrane in cells missing Osh4p/Kes1p. Consistent with this, it has been found that PI(4)P levels are elevated approximately 1.8-fold in cells lacking this protein and that this increase is largely dependent on Pik1p (Fairn et al. 2007). Whether Osh4p/Kes1p actually directly inhibits Pik1p activity in vitro or in cells has not yet been determined.

McMaster and coworkers have proposed an alternate model for Osh4p/Kes1p function: They suggest it binds PI(4)P on Golgi membranes and regulates the access of other proteins to this critical lipid (Fairn et al. 2007). Thus, the low level of PI(4)P made in *pik1^{ts}* cells at the nonpermissive temperature is sufficient for vesicular trafficking when Osh4p/Kes1p is absent because PI(4)P-binding proteins do not have to compete with Osh4p/Kes1p for access to this lipid. Whether Osh4p/Kes1p binds PI(4)P in the Golgi complex with a high enough affinity for this model to be correct remains to be determined. It is also not clear

what, if any, role sterol binding by Osh4p/Kes1p would play in either of the two models of its function at Golgi membranes.

The finding that Osh4p/Kes1p (and other ORPs) has multiple membrane binding surfaces (Schulz et al. 2009) suggests an alternate model of how it might play a direct role in the formation of Golgi transport vesicles. The creation and fission of vesicles require the transient generation of highly negatively curved membranes. Some lipids, such as PIPs and sterols, promote or inhibit the formation of these membrane structures (Bard & Malhotra 2006, Chernomordik & Kozlov 2003, De Matteis & Godi 2004). Therefore, proteins that alter the distribution of lipids within a membrane could change the propensity of the membrane to form the highly curved intermediates that occur during membrane vesicle formation and fusion. The multiple membrane-binding surfaces on Osh4p/Kes1p could help enrich this protein at highly negatively curved membranes on the TGN. The protein could then affect the rate of vesicle formation and fission by modulating the lipid composition of highly curved membrane domains by altering the sterol or PIP concentration in these domains.

Mammalian ORPs

Mammalian ORPs, like those of yeast, have been implicated in many functions. Here we focus primarily on their roles in signaling.

Signaling and OSBP—The extracellular signal-related kinase (ERK) signaling pathway plays many critical functions in mammals (Yao & Seger 2009). OSBP has been found to regulate the phosphorylation status of ERK, which affects its activity (Wang et al. 2005c). OSBP does so by binding with and regulating the activity of two protein phosphatases, serine/threonine phosphatase PP2A and tyrosine phosphatase PTPBS, enzymes that coordinately remove phosphates from ERK. Formation of this complex of OSBP with the phosphatases is regulated by sterols; depleting cells of cholesterol causes it to disassemble, as does the addition of 25-OH-cholesterol. Disassembly of the complex causes ERK to become hyperphosphorylated. Thus, OSBP functions as a sterol sensor that regulates ERK activity in response to binding sterols by functioning as a “scaffold” for the assembly of a signaling protein complex (Wang et al. 2005c).

Interestingly, the regulation of ERK by OSBP could help mediate changes in gene expression that occur in response to alterations in cellular cholesterol levels. Sterol response-element binding proteins (SREBPs) are transcription factors that control genes required for the synthesis and uptake of cholesterol (Espenshade & Hughes 2007). Because ERK is thought to affect the phosphorylation and activity of SREBP (Botolin et al. 2006, Kotzka et al. 2000, Kotzka et al. 2004), OSBP could affect SREBP activity in response to binding sterol.

OSBP has also been implicated in a second signaling pathway. The signal transducer and activator of transcription-3 (STAT3) is essential for the signal transduction of many cytokines and is regulated by Janus-activated kinase-2 (JAK2), which phosphorylates it (Leonard 2001). It has been demonstrated that JAK2 can phosphorylate OSBP and that STAT3 binds phosphorylated OSBP (Romeo & Kazlauskas 2008). When STAT3 binds OSBP, it can itself be phosphorylated in a JAK2-dependent manner and activated. This process was regulated by oxysterols. Thus, OSBP could function as a sterol-dependent scaffold in a second signaling pathway.

Cholesterol efflux and ORP2—ORP2 is expressed in all tissues but is most abundant in the brain (Laitinen et al. 2002). The protein is largely cytosolic but overexpression causes it to become enriched on Golgi membranes even though it lacks a PH domain. It binds

cholesterol and other oxysterols with nanomolar affinity (Hynynen et al. 2009). There is some evidence that ORP2 overexpression increases cholesterol efflux from cells (Hynynen et al. 2005, Hynynen et al. 2009, Laitinen et al. 2002). The increase in cholesterol efflux was accompanied by an upregulation of mechanisms cells use to compensate for cholesterol loss including increased cholesterol synthesis; increased uptake of low density lipoprotein (LDL), a major source of exogenous cholesterol; and decreased synthesis of cholesteryl esters. These findings suggest that as ORP2 promotes sterol efflux from cells, counterbalancing changes in cholesterol metabolism make up for the loss of cholesterol. In more recent work, it has been found that ORP2 partially localizes on lipid droplets (LDs) and that an ORP2 mutant defective in sterol binding is highly enriched on these organelles (Hynynen et al. 2009). LDs are sites of storage for the neutral lipids triacylglyceride and cholesteryl ester. Knockdown of ORP2 expression with RNA interference (RNAi) increases the amount of both lipids. Taken together, these findings suggest that ORP2 could play a role in neutral lipid metabolism and sterol trafficking, perhaps by functioning as a sterol sensor on LDs or by directly facilitating the movement of lipids to or from LDs.

ORPs and ATP-binding cassette transporter 1 (ABCA1)—One of the major routes of cholesterol efflux from cells is transfer to apolipoprotein AI (ApoAI), a process that requires the ATP-binding cassette transporter 1 (ABCA1) (Tall 2008). Recent evidence suggests that two ORPs may regulate ABCA1 expression and therefore cholesterol levels in cells; RNAi silencing of OSBP in Chinese hamster ovary (CHO) cells and J774 macrophages and of ORP8 in THP-1 macrophages increases the protein level of ABCA1 (Bowden & Ridgway 2008, Yan et al. 2008). In both cases, increasing ABCA1 levels are accompanied by elevated cellular cholesterol efflux to ApoAI. These findings indicate that ORPs are involved in maintaining cellular cholesterol homeostasis, but the mechanism by which reducing ORP levels causes an increase in the amount of ABCA1 in cells is not yet known.

Cholesterol transfer by mammalian ORPs—Recent evidence suggests that some mammalian ORPs, such as some yeast ORPs, can facilitate sterol transfer. Work from the Ridgway group has demonstrated that OSBP and ORP9L can transfer cholesterol between liposomes and that transfer is regulated by PI(4)P (Ngo & Ridgway 2009). Both proteins can localize to the Golgi complex, but their localization seems to be differently regulated. OSBP is largely cytosolic but moves to the Golgi complex in response to oxysterols (Ridgway et al. 1992), where it affects ceramide transport and sphingomyelin synthesis (Perry & Ridgway 2006). The Golgi complex localization of OSBP requires its PH domain, PI(4)P synthesized by the PI 4-kinase III- β (Balla et al. 2005), and interactions with other Golgi complex proteins including Arf and Nir2 (Levine & Munro 2002, Peretti et al. 2008). In contrast, ORP9L localizes to the TGN, but it does not colocalize with PI 4-kinase III- β , and its intracellular distribution is not affected by oxysterols (Ngo & Ridgway 2009). Whether either OSBP or ORP9L actually moves cholesterol into or out of TGN membranes in vivo remains to be determined. Knockdown of ORP9L by RNAi resulted in Golgi complex fragmentation, partial inhibition of cargo traffic from ER to Golgi complex, and a 50% increase in lysosomal and endosomal cholesterol as visualized by filipin staining. These findings suggest that ORP9L may be directly involved in cholesterol transport between the TGN and other compartments, perhaps the ER.

FUTURE DIRECTIONS

We are still just beginning to understand how ORPs function in cells. They have been implicated in many cellular processes, but much remains to be learned. For example, almost nothing is known about the role of ORPs in whole animals. In yeast and likely in higher

eukaryotes as well, cells express multiple ORPs with overlapping functions, which complicates studies.

The past few years have seen substantial progress in our understanding of how ORPs bind lipids and membranes, which appears to be surprisingly complex. It is now clear that even the core lipid-binding ORD found in all ORPs has multiple lipid and membrane-interacting surfaces. In addition, most ORPs have additional membrane and lipid-binding motifs outside the ORD. The major challenge for the future will be to begin to unravel how lipid and membrane binding by ORPs are integrated with their various functions. This will require reconstitution of ORPs together with the membranes and proteins with which they interact. It will also require the development of new techniques to better understand both how proteins interact with membranes and how ORPs might transiently affect the distribution of lipids in a membrane.

That many ORPs (and other lipid transport proteins) operate at organelle contact sites is an attractive hypothesis, but there is still little evidence to support it. Developing better methods to study organelle contact sites and identify proteins that localize to them will be critical for understanding how lipids and signals are transferred at these sites as well as what roles ORPs may play in this process.

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Figure 1.

Domain organization of the oxysterol-binding protein (OSBP)-related proteins (ORPs) from humans and *S. cerevisiae* is shown. The division of the human ORPs into groups, designated with Roman numerals, is taken from Lehto et al. (2001). FFAT, two phenylalanines in an acidic tract; GOLD, Golgi dynamics.

Figure 2. Phylogenetic tree of some ORPs. One of two parsimonious phylogenies of the ORPs is shown. Bootstrap support is indicated by branch thickness (*gray lines*), where thick branches represent bootstrap percentages >70%.

Figure 3. Structure of Osh4p/Kes1p. (a) Structure of the protein bound to cholesterol (*dark red*). The location of the lid domain, α -helix 7, and two distal membrane-binding loops are shown. (b) Structure of the protein in the absence of ligand. (c) Surface rendering of Osh4p in panel a indicating the positively charged (*blue*) and negatively charged (*red*) surface (created with PyMOL software). (d) As in panel c, but with the protein rotated 180° around the y axis. ALPS, ArfGAP1 lipid packing sensor.

Figure 4.

Possible mechanisms of ORPs. (a) Lipid transfer by an ORP between two closely apposed membranes. (b) Transient changes in lipid distribution in a membrane induced by ORP binding and/or lipid transfer. (c) ORPs could function as lipid sensors that alter signaling and/or their protein interaction partners in response to ligand binding. (d) ORPs could also present a lipid to a second lipid-binding protein or (e) prevent a lipid-binding protein from accessing a lipid in a membrane. VAP, vesicle-associated membrane protein-associated proteins; PH, pleckstrin homology.