Novel Members of the Human Oxysterol-binding Protein Family Bind Phospholipids and Regulate Vesicle Transport*

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Oxysterol-binding proteins (OSBPs) are a family of eukaryotic intracellular lipid receptors. Mammalian OSBP1 binds oxygenated derivatives of cholesterol and mediates sterol and phospholipid synthesis through as yet poorly undefined mechanisms. The precise cellular roles for the remaining members of the oxysterol-binding protein family remain to be elucidated. In yeast, a family of OSBPs has been identified based on primary sequence similarity to the ligand binding domain of mammalian OSBP1. Yeast Kes1p, an oxysterol-binding protein family member that consists of only the ligand binding domain, has been demonstrated to regulate the Sec14p pathway for Golgi-derived vesicle transport. Specifically, inactivation of the KES1 gene resulted in the inability of yeast to survive in the absence of Sec14p, a phosphatidylinositol/phosphatidylcholine transfer protein that is normally required for cell viability due to its essential requirement in transporting vesicles from the Golgi. We cloned the two human members of the OSBP family, ORP1 and ORP2, with the highest degree of similarity to yeast Kes1p. We expressed ORP1 and ORP2 in yeast lacking Sec14p and Kes1p function and found that ORP1 complemented Kes1p function with respect to cell growth and Golgi vesicle transport, whereas ORP2 was unable to do so. Phenotypes associated with overexpression of ORP2 in yeast were a dramatic decrease in cell growth and a block in Golgi-derived vesicle transport distinct from that of ORP1. Purification of ORP1 and ORP2 for ligand binding studies demonstrated ORP1 and ORP2 did not bind 25-hydroxycholesterol but instead bound phospholipids with both proteins exhibiting strong binding to phosphatidic acid and weak binding to phosphatidylinositol 3-phosphate. In Chinese hamster ovary cells, ORP1 localized to a cytosolic location, whereas ORP2 was associated with the Golgi apparatus, consistent with our vesicle transport studies that indicated ORP1 and ORP2 function at different steps in the regulation of vesicle transport.

Oxysterols are naturally occurring hydroxylated derivatives of cholesterol that can affect lipid homeostasis through both transcriptional and post-translational mechanisms (1–3). One such oxysterol, 25-hydroxycholesterol, was demonstrated to bind with high specificity to a low abundance cytosolic receptor termed oxysterol-binding protein 1 (OSBP1) (4). Human OSBP1 was predicted to encode a protein of 807 amino acids with an N-terminal glycine/alanine-rich region and PH domain and a C-terminal ligand binding domain. OSBP1 translocated from a cytoplasmic compartment of the Golgi apparatus when cells were treated with 25-hydroxycholesterol or in response to decreased plasma membrane cholesterol. The PH domain was required for translocation of OSBP1 to the Golgi, and this translocation directly correlated with alterations in sterol and phospholipid homeostasis through both transcriptional and post-translational mechanisms (5–7). The precise molecular mechanism by which OSBP transduces these signals is currently unknown.

In yeast, a family of OSBP proteins has been identified based on primary sequence similarity to the ligand binding domain of mammalian OSBP1 (8–10). Genetic studies on yeast have provided some clues toward understanding the biological function and mechanism of OSBPs. It was demonstrated the Kes1p, an oxysterol-binding protein family member that consists of only the ligand binding domain, was a negative regulator of the SEC14 pathway for Golgi-derived vesicle transport (11). Specifically, inactivation of the KES1 gene resulted in the ability of yeast to survive in the absence of Sec14p, a protein that is normally essential for cell viability (12–14). This regulation was specific to Kes1p as inactivation of the genes of the remaining members of the yeast OSBP gene family was unable to bypass the essential function of Sec14p. Sec14p is a phosphatidylinositol (PI)/phosphatidylcholine (PC) transfer protein, which in vitro catalyzed transport of phospholipid monomers from one membrane bilayer to another (14–16) and in vivo is speculated to function as a diffusible sensor of PC levels that in turn regulates lipid homeostasis for subsequent downstream management of Golgi-derived vesicle trafficking and sorting (11, 17–21). Besides KES1, inactivating mutations in several other structural genes have been demonstrated to relieve the cell of its essential requirement for Sec14p and these include the following: (i) each of the enzymes in CDP-choline pathway for PC biosynthesis, and (ii) Sac1p, a PI-4-phosphate phosphatase (22, 23). Additional experimentation demonstrated that in

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1 The abbreviations used are: OSBP, oxysterol-binding protein; ORP, oxysterol-binding protein-related protein; PC, phosphatidylcholine; PA, phosphatidic acid; PI, phosphatidylinositol; PI-4-P, phosphatidylinositol 4-phosphate; PI-4,5-P, phosphatidylinositol 4,5-bisphosphate; PI-3-P, phosphatidylinositol 3-phosphate; GFP, green fluorescent protein; CPY, carboxypeptidase Y; EST, expressed sequence tag; CHO, Chinese hamster ovary; PCR, polymerase chain reaction; PBS, phosphate-buffered saline.
increased expression of KES1 in sec14ts yeast containing inactivating mutations in any of the enzymes of the CDP-choline pathway reversed the sec14Δ bypass afforded by each of these mutations (11). Therefore, Kes1p likely counterbalances Sec14p function, with both genes normally required to prevent toxicity of the CDP-choline pathway and Sac1p to Golgi-derived vesicle transport.

To understand more precisely the functions and underlying mechanisms of mammalian OSBs, we screened a human cDNA library and isolated the two members of the human OSBP family that most resemble yeast Kes1p. We purified the encoded proteins and demonstrated that neither could bind 25-hydroxycholesterol but instead bound phosphatidic acid (PA) with high affinity and phosphatidylinositol-3-P (PI-3-P) with weak affinity. Thus, these new proteins were termed oxysterol-binding protein-related proteins, ORP1 and ORP2. Expression of ORP1 in yeast reversed the bypass provided by inactivation of the KES1 gene in cells lacking a functional Sec14p, whereas ORP2 expression was unable to do so. Instead, overexpression of ORP2 resulted in the cessation of cell growth in wild type yeast, and this correlated with a block in vesicle transport within the Golgi. ORP2-mediated cell growth inhibition was more extreme in cells lacking a functional Kes1p implying Kes1p/ORP1 shares a biological function with ORP2. Expression of ORP1- and ORP2-green fluorescent protein (GFP) chimera in Chinese hamster ovary (CHO) cells localized ORP1 to a cytosolic compartment and ORP2 to the Golgi apparatus.

**EXPERIMENTAL PROCEDURES**

**Media and Reagents—** Standard molecular biology methods, yeast genetic techniques, and transformation methods were used (24, 25). Yeast complex medium supplemented to a final glucose concentration of 2% (YPD) and synthetic minimal media have been described (25). Tran[^35]S-labeled methionine/cysteine mixture was purchased from PerkinElmer Life Sciences. Goat anti-rabbit and anti-mouse conjugated antibodies were from Bio-Rad. The enhanced chemiluminescence kit was from Amersham Pharmacia Biotech. All phospholipids were products of Avanti Polar Lipids, except the phosphoinositides that were purchased from both Matreya and Biomol. Similar results were obtained using lipids from either source. Pure protein kinase Cα was purchased from Biomol.

**Yeast Strain Construction—** The yeast wild type strains w303-1a (a ura3-1 his3-11, 15 leu2-3, 112 trpl-1 ade2-1 can1-100), w303-1A (a ura3-1 his3-11, 15 leu2-3, 112 trpl-1 ade2-1 can1-100), and sec14ts (a ura3-52 his3-200 lys2-801 sec14 ts) have been described (25). Tran[^35]S-labeled methionine/cysteine mixture was purchased from PerkinElmer Life Sciences. Goat anti-rabbit and anti-mouse conjugated antibodies were from Bio-Rad. The enhanced chemiluminescence kit was from Amersham Pharmacia Biotech. All phospholipids were products of Avanti Polar Lipids, except the phosphoinositides that were purchased from both Matreya and Biomol. Similar results were obtained using lipids from either source. Pure protein kinase Cα was purchased from Biomol.

**Isolation of a Full-length Human ORP1 and ORP2 cDNAs—** A tBLASTn search of the expressed sequence tag (EST) data base revealed several groups of cDNAs that were similar to yeast Kes1p and human OSBP1. Two of these groups consisted of cDNAs predicted to contain only the ligand binding domain. One group contained only partial cDNAs, and a full-length ORP1 cDNA was isolated from an oligo(dT)-primed human brain cDNA library (Life Technologies, Inc.) using the Gene Trapper positive selection system (Life Technologies, Inc.) and a 25-mer oligonucleotide was synthesized based on the most 5’ EST DNA sequence, and this oligonucleotide was used to enrich for novel ORP1 cDNAs. A total of 1000–2000 clones from the enriched cDNA library were screened using a 32P-labeled oligonucleotide designed from the partial cDNA sequence. Positive clones with large inserts were subsequently sequenced by automated dye-termination DNA sequencing. A full-length ORP1 cDNA was isolated subsequently to

several rounds of enrichment and colony hybridization using oligonucleotides designed from the emerging upstream novel DNA sequences. The second OSBP homologue that contained only the ligand binding domain, ORP2, was obtained full-length from the Kazusa DNA Research Institute (Kisarazu, Japan).

**Construction of ORP1 and ORP2 Expression Vectors—** The full open reading frames of human ORP1 and ORP2 were amplified by PCR using Vent DNA polymerase (New England Biolabs). After additional incubation with Taq DNA polymerase (Life Technologies, Inc.) to add deoxyadenosine tails, the PCR products were subcloned into pcDNA2.1-Topo (Invitrogen). The integrity of the entire ORP1 and ORP2 amplified sequences was confirmed by DNA sequencing. The open reading frames were subsequently subcloned into the yeast pESC-URA expression vector (Invitrogen). The pESC-URA vector contains the GAL1 promoter upstream of the open reading frames for regulated expression of human ORP1 and ORP2 proteins by substituting galactose for glucose as carbon source. The open reading frames were also subcloned into the mammalian expression vector pEGFP-N1 (CLONTECH) resulting in the addition of the coding sequence for GFP in frame with the 3’ end of ORP1 and ORP2. The open reading frames were also subcloned into the Escherichia coli expression vector pET23b (Novagen) resulting in the addition of a His6 tag to the C terminus of the expressed protein.

**Yeast Growth Assays—** For the yeast growth assay, a single colony from the yeast strain was grown over night in media containing the appropriate nutrients to ensure plasmid maintenance. Yeast cell concentration was estimated by measuring absorbance at 600 nm, and the number of cells was removed from each culture. These cells were washed in minimal media containing 2% galactose substituted as carbon source; a series of 1:4 dilutions were made, and 1 μl of each dilution was spotted onto minimal media agar plates containing glucose and/or galactose and the appropriate nutrients to ensure plasmid maintenance. Cells were incubated at either 25 or 37 °C for 3–4 days.

**Carboxypeptidase Y (CPY) Processing Assay—** The CPY assay was carried out essentially as described (27). Yeast cells were grown in defined media containing the required nutrients to ensure plasmid maintenance, but 2% galactose was substituted as carbon source (to induce human ORP1 or ORP2 expression). Cells were back inoculated to an absorbance at 600 nm of 0.4 and grown for 6–8 h. Aliquots of cells (absorbance at 600 nm of 3.0) were resuspended in fresh media and incubated at 37 °C for 60 min. Cells were then labeled with [35S]methionine/cysteine for 10 min, and then unlabeled methionine and cysteine were added to the reaction media (subsequent to 10 min of radiolabeling) and incubated for 90 min. Cells were then treated with 1% Triton X-100 to solubilize the cell membrane and lysed with lysozyme and purified using glutathione resin as directed by the manufacturer, and the purified fusion protein was used for antibody generation. ORP2 antibodies were raised against the peptide CQERRGDHLRKAKLDEDSGKADSD coupled to keyhole limpet hemocyanin (New England Peptide). The purified GST-ORP1 protein chimera and the ORP2-coupled peptide were injected into rabbits sub-
cutaneously by the Dalhousie University Carleton Animal Care staff for the production of ORP1 and ORP2 antibodies.

Lipid Ligand Binding Assay—Pure ORP1 and ORP2 proteins and COS-7 extracts of overexpressed recombinant proteins were analyzed for 25-hydroxycholesterol binding exactly as described (4). Standard phospholipid binding assays were performed as described (29) by immobilizing 100 pmol of pure phospholipid on Hybond-C nitrocellulose membranes (Amersham Pharmacia Biotech). Blots were blocked with Tris-buffered saline containing 3% fatty acid-free bovine serum albumin. Pure protein preparations of ORP1, ORP2, or protein kinase Ca (10 pmol/ml) were allowed to bind to the immobilized lipids by incubating the lipid blots with the purified proteins in the presence of Tris-buffered saline containing 3% bovine serum albumin at 4°C for 12 h. Blots were washed with blocking buffer containing 0.1% Tween 20 (w/v) and incubated with ORP1, ORP2, or protein kinase Ca primary antibodies (1:1000) in blocking buffer for 1 h at room temperature, washed twice, and incubated for 1 h with secondary antibodies (1:10,000) coupled to horseradish peroxidase, washed six times with blocking buffer containing 0.1% Tween 20 (w/v), and subsequently developed using the ECL system (Amersham Pharmacia Biotech).

Cell Culture, Transfections, and Fluorescence Microscopy—CHO-K1 cells were cultured in Dulbecco’s modified Eagle’s medium containing 5% fetal calf serum and 34 µg/ml proline. Cells were seeded onto glass coverslips and transfected with pEGFP-ORP1 or pEGFP-ORP2 using LipofectAMINE (Life Technologies, Inc.). Thirty hours after transfection, cells were fixed in 10% sodium phosphate (pH 7.4), 225 mM NaCl, and 2 mM MgCl2 (PBS) containing 3% (w/v) formaldehyde for 15 min at room temperature. Following two washes in PBS containing 5 mM ammonium chloride, cells were permeabilized in PBS containing 0.05% (v/v) Triton X-100 for 10 min at room temperature, washed twice with PBS containing 1% (w/v) fatty acid-free bovine serum albumin, and incubated for 15 min at room temperature in PBS containing 1% fatty acid-free bovine serum albumin. To stain the Golgi apparatus, cells were incubated in PBS containing 1% fatty acid-free bovine serum albumin containing 10 µg/ml Lens culinaris (8) lectin coupled to Texas Red (EY Laboratories) for 1 h at room temperature. Cells were washed twice with PBS containing 1% fatty acid-free bovine serum albumin and mounted on microscope slides with 90% glycerol, 50 µM Tris-HCl (pH 9.0), and 2.5% 1,4-diazadicyclo(2,2,2)-octane. To stain the endoplasmic reticulum, cells were incubated in PBS containing 1% fatty acid-free bovine serum albumin containing mouse monoclonal antibodies to protein disulfide isomerase (Stressgen) at a 1:500 dilution followed by a goat anti-mouse secondary antibody coupled to Texas Red (1:4000). To disrupt the Golgi apparatus, cells were treated with brefeldin A (2 µg/ml) for 30 min prior to the fixation step.

RESULTS

Isolation of Human ORP1 and ORP2 cDNAs—The KES1 gene of yeast had been previously isolated based on the observation that inactivating mutations of KES1 resulted in cells that were able to bypass the essential requirement of a PC/PI transfer protein, Sec14p (17). Sec14p is necessary for vesicle trafficking competence from the late Golgi to the cell surface and yeast vacuole (functional equivalent of the mammalian lysosome) (12–14). This role in vesicle trafficking was demonstrated to be specific to Kes1p as genetic inactivation of the other members of the yeast OSBP family were unable to relieve cells of the requirement for a functional Sec14p (11). The only characterized mammalian OSBP is OSBP1, a protein that consists of a ligand binding domain similar to that of Kes1p but also has a large N-terminal extension containing a PH domain, a Gly/Ala-rich region. OSBP1 translocates to the Golgi apparatus from a cytoplasmic vesicular compartment and has been demonstrated to regulate flux through the sterol and phospholipid biosynthetic pathways through as yet unknown mechanisms (3, 5–8).

We searched the human expressed sequence tags (EST) data base using sequences from the ligand binding region of human OSBP1 and yeast Kes1p for mammalian ESTs that were predicted to contain only the ligand binding domain portion of the OSBP family. Two different classes of OSBP encoding cDNAs were identified. An alignment of one class of EST sequences with similarity to the ligand binding region of OSBP1 and Kes1p demonstrated that there were no full-length cDNAs represented. We employed a positive cDNA selection system to screen libraries for a full-length human cDNA. After several rounds of library enrichment, colony hybridization, restriction analysis, and cDNA walking, we isolated a new member of the OSBP family of proteins that we termed ORP1 (OSBP-related Protein, GenBankTM accession number AF274714). DNA sequencing revealed an insert of 3.3 kilobase pairs in size that contained an internal 1.3-kilobase pair open reading frame. Stop codons were found in all three reading frames upstream of the predicted initiator Met codon. The second member of the OSBP family that contained only the ligand binding domain contained ESTs that were predicted to be full length. One of these was obtained from the Kazusa DNA Research Institute (Kisarazu, Japan) and was sequenced in its entirety (GenBankTM accession number AF274714).

The ORP1 open reading frame codes for a protein of 437 amino acids with a calculated molecular mass of 46 kDa, whereas ORP2 predicts a 468-amino acid protein with a molecular mass of 53 kDa (Fig. 1). The proteins do not contain any predicted membrane spanning domains, nor were there any
recognizable sorting sequences or functional motifs.

Ability of ORP1 and ORP2 to Reverse kes1−-mediated Bypass of the sec14ts Cell Growth Phenotype—SEC14 is an essential yeast gene that codes for a PI/PC transfer protein necessary for vesicle trafficking from the late Golgi to the cell surface and the vacuole (lysosome) (12–14, 17–21). Yeast cells with a temperature-sensitive SEC14 allele (sec14ts) grow normally at 25 °C but die at the restrictive temperature of 37 °C (17). The requirement of SEC14 for cell survival can be bypassed by inactivating the KES1 gene (11, 17), and hence sec14ts kes1− yeast can grow at 37 °C due to the reestablishment of secretory competence, although the precise mechanism by which loss of Kes1p function can accomplish sec14− bypass has yet to be identified.

The ORP1 and ORP2 open reading frames were placed under control of the GAL1 promoter. This results in repression of transcription from the GAL1 promoter when yeasts are grown on glucose-containing media, but upon switching to galactose-containing media the promoter is derepressed and protein expression occurs. The GAL1::ORP1 and GAL1::ORP2 plasmids were introduced into wild type and sec14ts kes1::HIS3 yeast cells. The expression level of the ORP1 and ORP2 proteins correlated with the amount of galactose present in the medium with highest protein levels, as detected by Western blot, in cells grown in 85–100% galactose as carbon source (Fig. 2C). We were unable to obtain Western blot data for ORP2 expression in 100% galactose as overexpression of ORP2 at this level resulted in the cessation of cell growth (Fig. 2A). However, the expression level of ORP2 grown in 85% galactose was similar to that of ORP1 grown in 100% galactose, so comparisons of their ability to complement loss of function of yeast KES1 could be performed.

If ORP1 or ORP2 could complement KES1 then expression of ORP1 or ORP2 in sec14ts kes1− cells would result in cells that could no longer survive at 37 °C, the non-permissive temperature for the sec14ts allele. We observed that sec14ts kes1::HIS3 yeast cells transfected with ORP1 grew normally at 25 °C but did not grow at 37 °C whereas those expressing ORP2 (grown on 85% galactose) grew at both 25 and 37 °C (Fig. 2A and B). To ensure that expression of ORP1 did not confer a temperature-sensitive growth defect in and of itself, ORP1 was expressed in wild type yeast cells. The wild type cells grew normally at both 25 and 37 °C (Fig. 2). These results indicated that ORP1 expression reversed the bypass of the sec14ts allele afforded by inactivation of the KES1 gene and thus provided evidence that ORP1 may be the mammalian functional counterpart to yeast Kes1p.

Another interesting observation from these studies was that the cessation of cell growth due to high level overexpression of ORP2 in yeast (grown on 100% galactose) was more apparent in yeast that contained a non-functioning KES1 gene even at 25 °C, a temperature where Sec14p is functioning. This enhancement of phenotype in cells lacking a functional Kes1p implies that Kes1p/ORP1 and ORP2 likely affect a similar biological function.

ORP1 and ORP2 Affect Carboxypeptidase Y (CPY) Processing and Transport—To test if the ORP1- and ORP2-dependent growth phenotypes influenced Golgi-derived vesicle transport and to ensure that the alteration in growth observed upon ORP1 expression in sec14ts kes1::HIS3 yeast was indeed due to re-establishment of vesicle transport, we examined the effect of ORP1 and ORP2 expression on CPY protein processing. Normally, newly synthesized CPY protein is modified by N-glycosylation in the endoplasmic reticulum (P1 form) and is subsequently transported to the Golgi where CPY is further modified by core glycosylation (P2 form). The P2 form of CPY traverses the Golgi and is packaged into a vesicle destined for the vacuole (lysosome) where it is processed to its mature form.

In wild type yeast cells, after a 60-min chase of [35S]labeled methionine/cysteine into CPY, most of the labeled CPY protein was fully processed to the mature form (Fig. 3). In contrast, when the sec14ts cells were shifted to the non-permissive temperature of 37 °C, there was a marked accumulation of the P1 and P2 forms of the CPY protein, indicating decreased CPY processing and thus decreased vesicle transport. The normal CPY secretion pattern can be restored by a mutation in the kes1 gene in the sec14ts yeast (sec14ts kes1::HIS3) (Fig. 3). Expression of ORP1 in sec14ts yeast containing the inactivated kes1− gene resulted in an inhibition of CPY secretion and a re-institution of the block in vesicle transport provided by the sec14ts allele, consistent with our observation that ORP1 also complemented KES1-dependent growth defects (Fig. 2). To en-
Yeast cells were grown in defined media containing the required nutrients to ensure plasmid maintenance with 100% galactose as carbon source (to induce human ORP1) and 85% galactose, 15% glucose to induce ORP2 expression. Cells were back-inoculated to an absorbance at 600 nm of 0.4 and grown for 6–8 h. Aliquots of cells (absorbance at 600 nm of 3.0) were resuspended in fresh media and incubated at 37 °C for 60 min. The indicated yeast strains were grown overnight in defined media containing glucose as carbon source and with appropriate nutrients to ensure plasmid maintenance. Yeast cell concentration was estimated by measuring absorbance at 600 nm, and identical numbers of cells were removed from each culture. Cells were washed twice with minimal media containing 2% galactose, and yeast cells were labeled with [35S]methionine/cysteine for 10 min and then chased with 50 μM unlabeled methionine/cysteine for 0–60 min. CPY immunoprecipitation and detection was performed as described under "Experimental Procedures." P1 represents the ER form of CPY; P2 represents the Golgi form; and M is the mature vacuolar CPY.

As we had observed that induced overexpression of ORP2 (using 100% galactose) eventually resulted in cessation of cell growth, we tested whether CPY processing was blocked upon induction of ORP2 expression. Indeed, overexpression of ORP2 resulted in a marked delay in CPY processing from its P1 and P2 forms to its mature form (Fig. 4). This is consistent with the hypothesis that Kes1p/ORP1 and ORP2 affect the same biological process, in this case vesicle trafficking, that was derived from our observation that cell growth inhibition due to overexpression of ORP2 was more pronounced in cells lacking functional Kes1p/ORP1.
Purification of ORP1 and ORP2 Proteins and an Assessment of Their Lipid Ligands—The lipid binding specificity for members of the OSBP protein family has only been determined for mammalian OSBP1 (4, 28). The lipid binding domain of OSBP1 binds oxygenated derivatives of cholesterol with a preference for 25-hydroxycholesterol. We expressed His₆-tagged versions of ORP1 and ORP2 in E. coli and purified the proteins using metal affinity column chromatography. We were unable to detect binding to 25-hydroxycholesterol using the pure protein or the protein overexpressed in COS-7 or CHO cells (4). However, this was not entirely unexpected as inactivating or overexpressing the KES1 gene did not affect sterol metabolism, and altering rates of sterol metabolism were unable to affect cell growth or vesicle trafficking in an Sec14p-dependent manner. Phospholipid metabolism is intimately linked with Sec14p function as Sec14p itself is a PI/PC-binding protein, and all of the other known genes that alter growth and vesicle trafficking in yeast lacking a functional Sec14p code for proteins that alter the phosphate composition of polyphosphate phosphoinositols (13, 14, 17–23). Thus, we examined whether ORP1 or ORP2 could bind various phospholipids (Figs. 5A and 6) (29). Lipids were spotted onto nitrocellulose filters, and either pure ORP1 or ORP2 was added. Antibodies to ORP1 and ORP2 were used to detect protein binding to specific lipids. Both ORP1 and ORP2 strongly bound PA and weakly bound cardiolipin and PI-3-P (Figs. 5A and 6). Others (29) have observed that this method agrees with the phospholipid binding specificity observed using mixed micelle and liposome protocols. We included protein kinase Ca as a specificity control (30, 31), and we observed the expected high degree of binding versus phosphatidylycerine, with weaker binding toward PI-4,5-P₂, and little to no binding toward either PA or PC (Fig. 5B).

Intracellular Location of ORP1 and ORP2—Mammalian OSBP1 has been localized by immunofluorescence and was found in a vesicular cytoplasmic compartment. OSBP1 can be induced to translocate to the Golgi apparatus upon treatment of cells with 25-hydroxycholesterol (8) or by removal of cholesterol or sphingomyelin from the plasma membrane of cells in

FIG. 6. Concentration dependence of ligand binding by purified ORP1 and ORP2 proteins. A, substrate concentration dependence of ORP1 and ORP2 binding to phospholipids. The 1st lane on the left was spotted with 150 pmol of each lipid, and subsequent lanes contain 1:1 serial dilutions such that the last lane contains 0.08 pmol/ml of purified protein. Phospholipid amount used in each assay was 150 pmol. Phospholipids were immobilized on nitrocellulose membranes, and the membranes were blocked with Tris-buffered saline containing 3% fatty acid-free bovine serum albumin. Pure protein preparations of ORP1 or ORP2 were allowed to bind to the immobilized lipids by incubating the lipid blots with the purified proteins in the presence of 3% fatty acid-free bovine serum albumin. Protein amount used for the assay was 10 pmol/ml. Phospholipid metabolism is intimately linked with Sec14p function as Sec14p itself is a PI/PC-binding protein, and all of the other known genes that alter growth and vesicle trafficking in yeast lacking a functional Sec14p code for proteins that either (i) decrease FC synthesis, (ii) alter the turnover of FC via phospholipase D, or (iii) affect phosphatases and kinases that alter the phosphate composition of polyphosphate phosphoinositols (13, 14, 17–23). Thus, we examined whether ORP1 or ORP2 could bind various phospholipids (Figs. 5A and 6) (29). Lipids were spotted onto nitrocellulose filters, and either pure ORP1 or ORP2 was added. Antibodies to ORP1 and ORP2 were used to detect protein binding to specific lipids. Both ORP1 and ORP2 strongly bound PA and weakly bound cardiolipin and PI-3-P (Figs. 5A and 6). Others (29) have observed that this
culture (3, 5–7). Yeast Kes1p has yet to be localized through microscopic means but has been localized through subcellular fractionation and was found in various particulate and soluble subcellular fractions (11). Human ORP1 and ORP2 were expressed in CHO cells as chimeras with GFP to assess their subcellular location. Western blots using GFP antibodies indicated full-length ORP1-GFP, and ORP2-GFP chimeras were made, and there was no sign of proteolytic products (data not shown), so the signal observed through microscopy is not due to partial chimeras or free GFP. Treatment of cells with brefeldin A, which collapses the Golgi into the endoplasmic reticulum, resulted in the relocation of ORP2-GFP but not ORP1-GFP, implying ORP2 is Golgi-localized (Fig. 7A). Consistent with this result was the overlapping immunofluorescence of ORP2 with the Golgi-specific L. culinaris lectin (Fig. 7B). The GFP-ORP1 chimera was mainly found diffused in the cytosol and was occasionally found in small amounts in the nucleus but did not localize with the endoplasmic reticulum marker protein disulfide isomerase (Fig. 6B).

**DISCUSSION**

OSBP1 is the prototypical member of a family of proteins that share a common ligand binding region near their C termini. Some members of the OSBP protein family consist almost entirely of the ligand binding region, whereas others possess N-terminal extensions containing a variety of motifs including PH domains, ankyrin-binding motifs, and a Gly/Ala-rich region (8, 10, 11). OSBP1 contains an N-terminal PH domain and Gly/Ala-rich region. OSBP1 has been cloned, purified, and demonstrated to bind oxygenated derivatives of cholesterol with varying degrees of efficiency (4, 8, 28). Upon ligand binding, OSBP1 translocates from a cytosolic compartment to the Golgi apparatus (3, 5–8, 33). Translocation of OSBP1 to the Golgi apparatus is associated with pleiotropic alterations in the metabolism of several lipids (3, 5–8), although the precise mechanism by which OSBP1 affects lipid metabolism as well as how these alterations affect the biology of the cell are still unclear. Work in yeast has indicated that one member of the OSBP family, encoded by the yeast KES1 gene, may participate in vesicle trafficking (11). A conditional lethal temperature-sensitive allele of SEC14 (sec14TS), an essential PC/P1 transfer protein required for vesicle transport from the Golgi, was used to search for genes whose inactivation resulted in the ability of cells to restore Golgi-derived vesicle transport and the associated reparation of cell growth when challenged with a nonfunctioning Sec14p (17). Inactivating mutation in several yeast genes was found to bypass the requirements for SEC14 and to date these include the following: (i) a yeast member of the OSBP family, KES1, (ii) each of the enzymes for PC synthesis through the CDP-choline pathway, and (iii) a PI-4-P phosphatase encoded by SAC1 (17, 22, 23). No other member of the yeast OSBP family was able to bypass the normally essential requirement for SEC14 indicating this ability is specific to KES1 function (11).

In our study we report the isolation of two novel human cDNAs, ORP1 and ORP2, coding for new members of the mammalian OSBP protein family. Similar to yeast Kes1p, ORP1 and ORP2 encode proteins that consist almost entirely of an OSBP-like ligand binding domain and possess no other obvious motifs or targeting signals. Expression of human ORP1, but not ORP2, in yeast containing a sec14TS allele and an inactivated KES1 gene reconstituted the sec14TS phenotype resulting in cell death due to decreased ability to transport vesicles from the Golgi at the non-permissive temperature for the sec14TS allele. High level overexpression of ORP2 in yeast resulted in growth cessation, and the growth defect was more pronounced when the KES1 gene was inactivated implying that Kes1p/ORP1 and ORP2 affect a similar cellular process. Consistent with this prediction there is a strong correlation between ORP2 overexpression with an inability to facilitate vesicle trafficking, as monitored by CPY processing, along the Golgi-mediated pathway. Also consistent with a role for ORP1 and ORP2 in vesicle trafficking through the Golgi pathway were our immunofluorescence experiments that position ORP2 in the Golgi apparatus and ORP1 in a cytosolic compartment.

ORP1 and ORP2 were purified, and ligand binding studies were unable to demonstrate binding to sterols (4, 8, 28). This was not entirely surprising as the sterol pathway in yeast does not alter known Kes1p-mediated events, and inactivation of the KES1 gene did not alter sterol synthesis in yeast (11). However, phospholipid metabolism is intimately associated with Kes1p biology, so ORP1 and ORP2 binding to a variety of phospholipids was tested. Both ORP1 and ORP2 strongly bound PA, with weak binding toward CL and PI-3-P also observed. PA is the product of phospholipase D, an enzyme that affects SEC14-mediated transport from the Golgi in yeast (21, 34) and appears to regulate vesicle formation in mammalian cells (35–37). Genetic evidence in yeast indicates that the regulation of vesicle transport from the Golgi by SEC14 is negatively regulated by phospholipase D and possibly KES1/ORP1. Our observation that human ORP1 and ORP2 have the capacity to bind PA implies that this binding may be an important requirement for their regulation of vesicle trafficking from the Golgi. ORP1 and ORP2 also bound the lipids CL and PI-3-P, although with a much lower affinity than for PA. The CL binding is likely not associated with in vivo ORP1 and ORP2 function as this lipid is found almost exclusively in the mitochondria, and there are no known links between ORP1, ORP2, KES1, or SEC14 and transport to the mitochondria. However, the PI-3-P binding may be associated with ORP1 and ORP2 phenotypes as the synthesis of PI-3-P is required for vacuolar targeting from the Golgi in yeast, and its conversion to PI-3,5-P is required to maintain appropriate vacuole size (32, 39). The goal of future studies will be to determine what ligand binding requirements are necessary for ORP1 and ORP2 to fulfill their roles in vesicle trafficking and cell growth regulation.

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