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Sterol-Dependent Nuclear Import of ORP1S Promotes LXR Regulated Trans-Activation of APOE

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Abstract

Oxysterol binding protein related protein 1S (ORP1S) is a member of a family of sterol transport proteins. Here we present evidence that ORP1S translocates from the cytoplasm to the nucleus in response to sterol binding. The sterols that best promote nuclear import of ORP1S also activate the liver X receptor (LXR) transcription factors and we show that ORP1S binds to LXRs, promotes binding of LXRs to LXR response elements (LXREs) and specifically enhances LXR-dependent transcription via the ME.1 and ME.2 enhancer elements of the apoE gene. We propose that ORP1S is a cytoplasmic sterol sensor, which transports sterols to the nucleus and promotes LXR-dependent gene transcription through select enhancer elements.

Keywords

ORP1; ORP1S; oxysterol; LXR; nuclear import; NLS

Introduction

Oxysterol binding protein related protein 1 (ORP1) belongs to a family of sterol-binding proteins with diverse functions [1]. The domain structure of these proteins consists of a variable N-terminal region, a sterol/oxysterol binding domain (OBD), a signature sequence and a coiled-coil domain. The N-terminal regions of different family members contain lipid and protein binding motifs such as pleckstrin homology (PH) domains, leucine zippers, VAP binding FFAT sequences and ankyrin repeats. These N-terminal elements target different ORP family members to different cellular locations. The sterol-binding domain allows all ORP family members to bind cholesterol and oxysterols, though affinity differences have

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been identified for different ORP family members [2-8]. The structure of the sterol-binding domain of a yeast ORP, Osh4p, has been solved and shows that sterols bind in a deep pocket that largely prevents solvent access to the sterol when bound [9]. The majority of protein contacts made by Osh4p to bound sterol are to features that are common to most sterols and this mechanism likely explains why ORP proteins can bind both cholesterol and oxysterols. The signature sequence is highly conserved, but has unclear function. The coiled coil domain is involved in protein interactions [6].

ORP family proteins are widely expressed from yeast to man and appear to function as sterol transporters. *S. cerevisiae* has 7 members ORP family members (Osh1p~ 7p) and deletion of all 7 members sharply reduces sterol transport between the plasma membrane and the ER [10-13]. Mammalian ORP proteins can also transport sterols [8, 14, 15]. ORP1S, whose small N-terminal domain lacks protein and phospholipid interaction modules, has been proposed to transport sterols through the cytosol [15]. Larger ORP proteins with N-terminal domains containing PH and FFAT domains may bridge between the ER and other cellular membranes to facilitate proximity-based transfer of sterols between cellular compartments [14-17].

ORP1 is expressed as two alternatively spliced isoforms (Fig 1) with highest expression in brain, heart, macrophages and skeletal muscle [18, 19]. Both the larger (ORP1L) and smaller (ORP1S) isoforms share a common 437 amino acid C-terminal sequence that encompasses the OBD, signature sequence and coiled coil domain. The larger isoform (ORP1L) has an additional 513 residue-long N-terminal extension that contains ankyrin repeats and a PH domain [18]. ORP1L localizes to late endosomes/lysosomes and may regulate the vesicular traffic in the endocytic pathway as a part of a complex with the small GTPase, Rab7, and its effector protein RILP [20, 21]. Overexpression of human ORP1L in mouse macrophages impairs cholesterol efflux and increases atherogenesis in LDL-receptor deficient mice [4]. By contrast, ORP1S shows diffuse staining and has been proposed to function as a cytosolic sterol carrier because ORP1S can complement Osh4 function in yeast [22] and can facilitate sterol transfer between the ER and plasma membrane [15].

The liver X receptor protein family (LXR) consists of two members, LXR and LXR, which function as nuclear receptors [23]. LXR is highly expressed in liver, adipose tissue, small intestine, and macrophages [24], whereas LXR is ubiquitously expressed [25]. LXRs form a complex with retinoid X receptor (RXR) on LXR response elements (LXRE) within the genome. Binding of LXRs to specific oxysterols strengthens the interaction of LXR/RXR with LXREs and induces the trans-activation activity of the LXR/RXR complex, thereby stimulating the expression of genes involved in lipid and cholesterol metabolism, glucose homeostasis, and inflammatory responses [26, 27]. The oxysterols that activate LXRs are hydroxylated metabolites of cholesterol; however, it is unclear whether oxysterols reach LXRs by diffusion or via a carrier protein. Other nuclear receptors that bind hydrophobic ligands use ligand carrier proteins to facilitate nuclear delivery of ligand. Examples include CREBPII and FABP, which transport retinoic acid and eicosinoids/fatty acids to RAR and PPAR / , respectively [28, 29]. The ability of ORPs to transport sterols suggests that ORPs may serve as ligand carriers for LXRs.

Here we report that ORP1S shuttles between the cytoplasm and the nucleus, binds to LXR α / β , and facilitates LXRE-driven trans-activation via specific enhancer elements. Sterol binding is required for migration of ORP1S from the cytoplasm to the nucleus where LXR / resides. We propose that ORP1S transports oxysterol ligands to LXR α / β in the nucleus and thereby facilitates the LXRE-driven trans-activation.

Materials And Methods

Materials

ExTaq DNA polymerase was from Takara Bio (Otsu, Shiga, Japan). Protoscript First Strand cDNA Synthesis kit, Taq 2X master mix and the restriction enzymes used in this manuscript were from New England Biolabs (Ipswich, MA). Eugene 6, Eugene HD and anti-GFP mouse mAb were from Roche Diagnostics (Indianapolis, IN). Anti-myc mouse mAb (clone 4A6) was from UBI (Lake Placid, NY). Anti-GFP rabbit pAb and anti-HA rabbit pAb were from Abcam (Cambridge, MA). Anti-HA mouse mAb, anti-Actin mouse mAb and all chemicals were from Sigma (St. Louis, MO) unless stated otherwise. Anti-STAT3 rabbit pAb, and HRP-conjugated anti-goat donkey IgG were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-protein disulfide isomerase pAb was from Stressgen Biotechnologies (San Diego, CA). Alexa Fluor-conjugated secondary antibodies, Hoechst 34580, pcDNA3.1 vectors, and anti-APP rabbit pAb were from Invitrogen (Carlsbad, CA). The HRP-conjugated anti-mouse and anti-rabbit goat IgG were from Bio-Rad (Hercules, CA). Phosphatase inhibitor cocktail set II, protease inhibitor cocktail set III and doxycycline HCl were from RPI (Mt. Prospect, IL). GSH sepharose was from Amersham (Piscataway, NJ). Sterols, except those indicated below, were from Steraloids Inc (Newport, RI). 24(S),25-epoxycholesterol, 24(S)-hydroxycholesterol and compactin were from Biomol (Plymouth Meeting, PA). 22(R)-[3H]-hydroxycholesterol was from American Radiolabeled Chemicals (St. Louis, MO). 1, 2-[3H]-(N)-cholesterol and 25-(26, 27-[3H]) hydroxycholesterol were from PerkinElmer (Boston, MA). Enhanced chemiluminescent (ECL) substrate was from Pierce (Rockford, IL). Hygromycin and blasticidin were from Invivogen (San Diego, CA).

Plasmid construction

Human ORP1S (NCBI GI:19718745) and ORP1L (NCBI GI:19718740) were cloned from a cDNA library of HeLa cells using PCR with 5' NheI and 3' HindIII sites and subcloned into the pGEM-T EZ TA cloning system (Promega, Madison, WI), resulting in pGEM-ORP1S and pGEM-ORP1L respectively. A 3' EcoRI site and 3' stop codon on the vector sequence were removed from pGEM-ORP1S and pGEM-ORP1L using QuikChange system (Stratagene, La Jolla, CA) for subcloning purposes. The cDNA of ORP1S was then subcloned from pGEM-ORP1S into pcDNA3.1(-)Myc-His using the 5' NheI and 3' HindIII restriction enzymes. The cDNA of ORP1L was subcloned into pcDNA3.1V5-His using the 5' NheI and 3' HindIII restriction enzymes. The cDNAs encoding C-terminal GFP fusion protein of ORP1S and ORP1L, designated pORP1S-EGFP and pORP1L-EGFP, were constructed by subcloning ORP1S and ORP1L into pEGFP-N3 using the 5' EcoRI and 3' SalI restriction sites. Bacterial expression plasmids for hLXRa-HA, hRXRa and hORP1S were constructed by cloning the respective cDNAs into pGEX4T-3. Mammalian luciferase transactivation plasmids were constructed by subcloning a PCR product consisting of the apoE proximal promoter (bp -651 to +83 [30]) into pGL3-Basic plasmid (Promega, Madison, WI) using the 5' BglII and 3' HindIII restriction sites to create pGL-651. The enhancer elements, ME.1, ME.2 and HCR1 were cloned by PCR from human genomic DNA with MluI sites at both ends and subcloned into the MluI site of pGL-651 to create pGL-ME.1-651, pGL-ME.2-651 and pGL-HCR1-651, respectively. LXRE sequence in ME.1 of pGL-ME.1-651 was mutated as previously described [31] to create pGL-ME.1mut-651 using QuikChange system (Stratagene, La Jolla, CA). The ME.1, ME.2, HCR1 and ME.1mut enhancer elements were subcloned into pTK-luciferase to create pME.1-tk, pME.2-tk, pHCR1-tk and pME.1mut-tk, respectively, using MluI.

Protein expression and purification

Bacterial expression of GST-ORP1S was induced by IPTG for 6 hours at room temperature. Bacteria were collected and resuspended in buffer A (pH 7.4, 20 mM HEPES, 150 mM

NaCl, 1% glycerol, 0.1% Triton X-100, 0.2% protease inhibitor cocktail III, and 1 mM phenylmethylsulfonyl fluoride) for sterol-binding assay or in lysis buffer (PBS with 0.1 % CHAPS, 1 mM PMSF (make 1000 × stock in DMSO first), 0.25 mg/ml lysozyme, 500 U/40 ml DNase I, 1 mM DTT, 10 μM MgCl₂) for ABCD assay (see below). Bacteria were lysed with a French press and centrifuged at 100,000 × g for 30 min to remove cell debris. The clarified supernatant fraction was subjected to glutathione affinity chromatography. The amount of purified protein immobilized on the beads was determined by the Bradford method. GST-hLXRα-HA and GST-hRXRα were expressed and purified as described previously [32].

Cell Culture

HeLa and HEK293T cells were maintained in medium A (DMEM supplemented with 10 % FBS, 100 U/ml penicillin G, and 100 μg/ml streptomycin sulfate). To deplete cells of cholesterol, cells were incubated in the presence of medium B (DMEM containing 5 % lipoprotein poor serum (LPPS), 100 U/ml penicillin G, 100 μg/ml streptomycin sulfate plus either 50 μM compactin and 50 μM mevalonate for HeLa cells or 10 μM compactin and mevalonate for HEK293T cells as described previously [16]). To acutely deplete cells of cholesterol, cells were incubated in medium A overnight and then changed to medium B containing 0.5 % methyl-β-cyclodextrin (MCD) for 1 hr. HEK293 cells stably expressing a tetracycline inducible HA-tagged LXRα or β (a kind gift from Dr. Joyce Repa at U.T. Southwestern Medical Center at Dallas, TX) was maintained in medium A containing 200 μg/ml hygromycin and 1 μg/ml blasticidin. To induce the expression of LXR-HA, cells were incubated in medium B for two days containing 2 μg/ml doxycycline.

Indirect immunofluorescence microscopy

Indirect immunofluorescence staining was performed on HeLa cells transiently expressing ORP1S-myc/His, ORP1L-V5/His or ORP1S-EGFP. On day 1, HeLa cells (1×10⁵ cells/ml) were seeded on glass coverslips in 6-well culture plates. On day 2, cells were transiently transfected with the indicated cDNA using Fugene 6 and incubated in medium A for 5 hr before changing to medium B for 12 hr to deplete the endogenous and exogenous source of cholesterol. Cells were treated with the indicated sterol in medium B for 2 hr and fixed with 4 % (w/v) paraformaldehyde in PBS (pH 7.4) for 15 min at RT. Following three washes in PBS, cells were permeabilized with 0.1 % (v/v) Triton X-100 in PBS for 5 min at 4°C. Cells were incubated with -myc mAb for 30 min at 37°C. After three washes in PBS, cells were incubated with Alexa 488 -mouse goat IgG for 30 min at 37°C. After the fluorescent antibody was removed, cells were incubated in Hoechst 34580 for 10 min to stain the nucleus, washed three times in PBS, and mounted on the slides using 10 % (w/v) Mowiol, 25 % (v/v) glycerol in 100 mM Tris (pH 8.5). Epifluorescence microscopy and confocal scanning microscopy were performed using either a Zeiss Axioplan 2E or a Lecia TCS SP5 microscope in Live Cell Imaging Core. To quantify the nuclear localization of ORP1S, images of HeLa cells expressing ORP1S-EGFP were acquired using a confocal scanning microscope and analyzed using NIH ImageJ 1.4. The mean fluorescence intensities of ORP1S-EGFP in the area defined by a circle with radius of 25 pixels were acquired from the nuclear and cytosolic areas. The ratio of nuclear to cytoplasmic fluorescence intensity (N/C) was measured from at least 50 cells for each condition, averaged and plotted with GraphPad Prism software. Error bars indicate ±SD. One-way ANOVA was applied for the comparison of multiple groups using Graphpad Prism software.

Live Cell Imaging

Live cell imaging was carried out on HeLa cells transfected with ORP1S-PAGFP. Cells (1×10⁵ cells/ml) were grown on 35-mm culture dishes with glass bottom (MatTek #P35G-1.0-20-C) and transfected with 1 μg of cDNA using Fugene 6. Cells were then

incubated in medium A or B for 12 hours and treated or not with 22R-hydroxycholesterol for 2 hrs. Photoactivation and confocal laser scanning microscopy on live cells were carried out on a Zeiss LSM 510 META confocal microscope equipped with CO₂ chamber at the Live Cell Imaging Core of University of Southwestern Medical Center. ORP1S-PAGFP or PAGAP were activated by two-photon excitation at 730 nm. Images were acquired before, immediately after and every 5 sec for 5 min following photoactivation. Fluorescence intensities within circles with radius of 40 pixels in cytosolic area and the nucleus were obtained using NIH ImageJ 1.4. Intensities were normalized to the pre-activation intensity and plotted using GraphPad Prism software.

Cell fractionation

Nuclear extract fractions were prepared as described previously [33] with minor modifications. Briefly, cells were scraped in cold PBS containing protease inhibitors, and centrifuged at $1000 \times g$ for 3 minutes. Harvested cells were resuspended in 4 volumes of hypotonic buffer C (pH 7.6, 10 mM HEPES, 10 mM NaCl, 0.5 mM EDTA, plus protease and phosphatase inhibitor cocktails) and incubated on ice for 15 minutes. Cells were lysed by 10 passages through a 22G needle. The lysed cells were then centrifuged at $1000 \times g$ for 7 min to separate the nuclei and unbroken cells from cytosol. The supernatant fraction was collected and designated the post-nuclear supernatant fraction (PNS). The pellet of nuclei and unbroken cells (~10 % unbroken cells) were resuspended in an equal volume buffer D (pH 7.6, 20 mM HEPES, 420 mM NaCl, 0.5 mM EDTA, 20 % glycerol, plus protease and phosphatase inhibitors) and incubated at 4°C with shaking for 20 min. Precipitable material was removed by centrifugation at $100,000 \times g$ for 30 min, leaving the supernatant fraction containing the nuclear extract, which was adjusted to 140 mM NaCl before use. Soluble fraction (S100) and total membrane fraction (P100) in PNS were separated by centrifugation at $100,000 \times g$ for 30 min.

Sterol binding assay

Sterol binding was performed as previously described [6] with minor modifications. Beads containing 50 pmol of GST-ORP1S were resuspended in 1 ml of buffer B (pH 7.4, 20 mM HEPES, 150 mM NaCl, 0.2 % ethanol and 1 mM PMSF) containing 1 mg/ml of BSA with the indicated concentration of [³H] labeled-sterol in a 1.5 ml microcentrifuge tube, and rotated for 2 hours at room temperature. Competitive binding of sterols to GST-ORP1S was assayed by incubating purified GST-ORP1S on beads with [³H]-sterol (10 nM) in the presence of the indicated concentration of unlabeled sterol in buffer B containing 1 mg/ml BSA. Following four washes at $1,000 \times g$ for 1 minute with 1 ml of buffer B, the bound GST fusion proteins were eluted with 50 mM reduced glutathione. The tritium radioactivity in the elution was then measured by liquid scintillation counting. Specific [³H]-sterol binding to GST-ORP1S was determined by subtracting the background radioactivity (amount bound to GST beads alone) from the total radioactivity. Each binding curve was plotted with GraphPad Prism software, and the apparent K_d determined using one-site-binding nonlinear regression analysis.

GST Pull-down assay

GST pull-down assays to determine whether ORP1S interacts with LXR / were performed by immobilizing recombinant GST-ORP1S or GST (50 pmol) on GSH beads and incubating these beads with nuclear extracts of HEK293T cells stably expressing HA-tagged LXR α/β in buffer B for 2 hr at room temperature with rotation. Following four spin-washes with buffer B, the bound proteins were eluted with 2X sample buffer, separated on SDS-PAGE and immunoblotted with the indicated antibody. HA-tagged LXR / was detected by α -HA mAb.

Avidin-biotin complex DNA binding assay (ABCD assay)

Avidin-biotin complex DNA (ABCD) binding assays were performed to measure DNA binding activity of LXR α -RXR α complexes to LXREs were performed as described previously [34]. Briefly, 1 μ g of double-stranded biotinylated oligonucleotide containing the LXREs of human apoE ME.1 enhancer (5'Biot/CCCACCAGCTGCCAGGGTCACTGGCGGTCAAAGGCAGCTGGTGG) or mutated ME.1 enhancer (5'Biot/CCCACCAGCTGCCAGGAACACTGGCGAACAAGGCAGCTGGTGG) was immobilized using 20 μ l Streptavidin agarose beads (Piercenet) and incubated with 200 ng of purified LXR α -HA and RXR α in the presence of 24S-HC loaded GST-ORP1S or GST-ORP1SP114L. The pre-loaded 24S-HC is the sole source of oxysterol. Following four spin-washes, the bound proteins were eluted with 2X SDS sample buffer, separated on SDS-PAGE and immunoblotted with the indicated antibody.

Immunoprecipitation and immunoblotting

The indicated proteins from nuclear extracts of HEK293T cells transiently expressing ORP1S-GFP and HA-tagged LXR α were immunoprecipitated as described previously [35]. Briefly, 2 mg of nuclear extract was pre-cleared by incubation with 50 μ l of protein A-agarose beads. After removing non-specifically bound proteins, the extract was then incubated in the presence of the indicated pAb at 4°C overnight. The antibody complex was isolated by incubating the antibody treated extract with protein A-beads for 2 hr at 4°C. Following four spin-washes with buffer B, the bound proteins were eluted with 2X SDS sample buffer, separated on SDS-PAGE and immunoblotted with the indicated antibody.

Luciferase reporter assay

LXRE-dependent transactivation was determined using a dual luciferase assay system. Briefly, HEK293 cells (2×10^5 cells/well) grown in 12-well plate were transfected with pCMX-LXR α (20 ng/well), pCMX-RXR α (20 ng/well), firefly luciferase reporter plasmid (200 ng/well), pRL-tk (200 ng/well), pcDNA-ORP1S-myc (400 ng/well) and pGEM (to a total of 1000 ng/well). After transfection, cells were incubated in medium B containing the indicated ligands or vehicle control for 24 hr. Firefly luciferase activity was measured as described previously [36] and normalized to Renilla luciferase activity (expressed from pRL-tk). The graphs shown are the averages of four independent experiments. Error bars indicate $\pm$ SD. Oneway ANOVA was applied for the comparison of multiple groups using Graphpad Prism software.

Results

Sterol-dependent nuclear import of ORP1S

Consistent with previous observations [3, 6], we found that ORP1S binds both cholesterol and oxysterols with high affinity (Fig 2A-F). ORP1S bound cholesterol with an estimated K_d of 393 nM, 25-HC with an estimated K_d of 167 nM, and 22R-HC with an estimated K_d of 96 nM. Our measurement for the K_d of ORP1S for 25-HC is similar to prior estimates of this association [3]. Competitive binding assays confirmed sterol-specific binding to ORP1S (Fig 2D, E, F). Random mutagenesis within ORP1S showed that the P114L mutation compromised high affinity binding of ORP1 to cholesterol, 25-hydroxycholesterol (25-HC), and 22R-HC (Fig 2A, B, C). P114 lies between the sterol binding domain and the signature sequence and is highly conserved between ORP paralogs and across species (Fig 1B, [3])

To determine whether the P114L mutation influenced the cellular distribution of ORP1S we transfected into HeLa cells expression plasmids encoding Myc-tagged ORP1S or ORP1S-

P114L and localized each ORP1S species by immunofluorescence. Consistent with previous findings [18], we observed that wild type ORP1S-Myc is diffusely distributed in both the nucleus and cytoplasm of cells grown in the presence of normal serum (Fig 2G); however, the sterol-binding defective ORP1S-P114L-Myc was excluded from the nucleus (Fig 2H). This observation suggested that sterol binding promotes ORP1S localization within the nucleus.

To test whether sterol binding is necessary for nuclear localization of ORP1S, we modulated sterol levels in cells. Sterol levels were depleted either by prolonged treatment with lipoprotein poor serum (LPPS), compactin and mevalonate or by acute treatment with methyl- β cyclodextrin. Both treatments sharply reduced the fraction of ORP1S-Myc that was present in the nucleus. Replenishment of cholesterol in LPPS/compactin/mevalonate treated cells using cholesterol loaded cyclodextrin (CL) restored nuclear localization of ORP1S-Myc. Introduction of epicholesterol, which does not bind well to the sterol binding domain of ORPs [6], was unable to restore nuclear localization of ORP1S-Myc in sterol depleted cells (Fig 3A). Quantification of the nuclear and cytoplasmic staining of ORP1S showed that serum and cholesterol increase the nuclear to cytoplasmic (N/C) ratio of ORP1S from 0.5 to 1.5 (Fig S2D). The effect of sterols on ORP1S was independent of the tag because ORP1S-GFP showed similar dependence on cholesterol for nuclear localization (Fig 3B). Expression levels do not appear to significantly influence the localization of ORP1S because the N/C was similar over a broad range of ORP1S expression (Fig S2B). The ability to bind sterol was required because neither serum nor cholesterol loaded cyclodextrin altered the cytoplasmic localization of ORP1S-P114L (Fig 3C). The cellular localization of ORP1L, which associates with late endosomes and lysosomes (Fig S2A and [18]) was unaffected by cellular sterol status (Fig 3D). Neither ORP1S expression nor the modulation of sterol levels had a general effect on trafficking of transcription factors between the cytosol and the nucleus because STAT3 localization did not change in response to cholesterol depletion or repletion in ORP1S-GFP transfected cells (Fig 3E). Consistent with a prior report [18], ORP1S did not pellet with cellular membranes either in the presence or absence of sterols, while ORP1L was present both in the soluble and membrane fractions of cell lysates (Fig S1). These observations suggest that sterol binding promotes ORP1S movement from the cytoplasm to the nucleoplasm.

To determine which sterols can drive nuclear import of ORP1S, we used confocal microscopy to compare the localization of ORP1S-GFP in the cells pre-treated with LPPS, compactin and mevalonate and then incubated with different sterols (Fig 4). We divided responses to sterols into three categories: non-stimulators, weak stimulators and strong stimulators. Non-stimulators included 7 α -HC, 5-androsten-3 β -ol, oleate, lanosterol, desmosterol, 7-dehydrocholesterol, ergosterol, β -sistosterol, stigmasterol, esterdiol, progesterone, 7 β -HC and 22S-HC. Weak stimulators included 25-HC and 27-HC. Strong stimulators included 20S-HC, 22R-HC, 24S-HC, and 24,25-epoxycholesterol (Fig 4).

Quantitative analysis of the strong stimulators showed that nuclear import was concentration-dependent (Fig 5A, B). Cells expressing ORP1S-GFP were cholesterol depleted by pre-treatment with LPPS, compactin and mevalonate and then cultured in the presence of increasing concentrations of sterols. The fluorescence intensity of the nucleus and cytoplasm at each concentration was measured and expressed as an N/C ratio. The nuclear localization of ORP1S-GFP was exquisitely sensitive to 22R-HC and 24S-HC with maximal effect reached by 2.5 μ M sterol (Fig 5A). 20S-HC, 25-HC and 24,25-epoxycholesterol also increased the N/C ratio (Fig 5B), but were substantially less potent than 22R-HC and 24S-HC. 5-androsten-3 β -ol, 7 α -HC and even 22S-HC, an optical isomer of 22R-HC, failed to stimulate nuclear import at all measured concentrations.

We hypothesized that the affinity of sterol binding to ORP1S might correlate with the ability of the sterol to drive nuclear import. To test this hypothesis we compared the ability of 22R-HC, 24S-HC and 22S-HC to compete with [³H]-25-HC for binding to ORP1S (Fig 5C, D). 22R-HC and 24S-HC, but not 22S-HC, strongly competed for binding to ORP1S (Fig 5C), while 20S-HC and 24,25-epoxycholesterol competed poorly with [³H]-25-HC for binding to ORP1S (Fig 5D). These observations indicate that sterol binding promotes nuclear localization of ORP1S.

To determine whether ORP1S is actively transported into the nucleus in response to sterol binding, we measured the rate of sterol-dependent nuclear import of ORP1S using photoactivatable GFP (PAGFP) tagged ORP1S (Fig 6). Addition of 22R-HC to ORP1S-PAGFP cells treated with LPPS, compactin and mevalonate caused ORP1S to enter the nucleus with a half time value ($t^{1/2}$) of 28.68 ± 3.5 seconds after photoactivation. By contrast, no measurable rate of ORP1S entry was observed in the absence of 22R-HC. PAGFP alone was observed in both the cytosol and the nucleus, but the rate of entry was not improved by 22R-HC addition ($t^{1/2}$ of 49 ± 11 seconds for no sterol as opposed to $t^{1/2}$ of 111 ± 13 seconds with 22R-HC). These results indicate that sterol binding to ORP1S is tightly associated with import into the nucleus and that ORP1S is actively transported into the nucleus upon sterol addition.

The rapid transport rate suggests that ORP1S uses a nuclear localization signal (NLS). ORP1S has three putative NLS sequences: ¹⁸KKHR²¹, ²⁵⁰KSKKK²⁵⁴, and ³⁸⁰KKRLEEKQRAARKNR³⁹⁴ [18]. We mutated each of these sequences and found that mutations of either ¹⁸KKHR²¹ to AAHA or ²⁵⁰KSKKK²⁵⁴ to AAAAA blocked the ability of ORP1S to enter the nucleus (Fig 7A, B). The KKHR sequence is near the sterol-binding site (Fig 1); however, the AAHA mutation did not compromise 22R-HC binding (Fig 7C). These results demonstrate that both ¹⁸KKHR²¹ and ²⁵⁰KSKKK²⁵⁴ sequences are required for nuclear import and that the activity of these NLSs are sensitive to sterol binding.

Sterol dependent nuclear import of ORP1S and LXR /

The strong affinity of 22R-HC and 24S-HC for ORP1S coupled with the ability of these sterols to drive the migration of ORP1S into the nucleus suggested that ORP1S facilitates the biological functions of these oxysterols in the nucleus. Because both 22R-HC and 24S-HC serve as activating ligands for LXR α/β [37], we tested whether ORP1S influences LXR function. To test whether ORP1S binds to LXRs, purified GST-ORP1S or GST alone was incubated with nuclear extracts of LXR-HA expressing (Fig 8A, lanes 1, 2 and 4, 5) or non-expressing (lane 3) HEK-293 cells. GST/GST-ORP1S bound material was then pulled down using glutathione agarose and LXR α -HA and LXR β -HA assayed by immunoblot. GST-ORP1S but not GST alone pulled down LXR (lane 4) and LXR β (lane 5). To verify these results, ORP1S-GFP or GFP alone were co-expressed in HEK-293T cells with LXR α -HA and immunoprecipitations performed with either anti-GFP IgG (Fig 8B, lanes 1,2) or non-immune IgG control (lane 3). Anti-GFP IgG co-precipitated LXR -HA with ORP1S-GFP (arrow, lane 2), but non-immune IgG did not (lane 3). GFP could not substitute for ORP1S-GFP (lane 1). In the reciprocal immunoprecipitation (Fig 8C), anti-HA IgG co-precipitated ORP1S-GFP in cells expressing both ORP1S-GFP and LXR α -HA (lane 2) but not in cells expressing ORP1S-GFP alone (lane 1). Non-immune IgG did not precipitate either ORP1S or LXR α (lane 3).

The interaction of ORP1S with LXR α/β suggested that ORP1S may promote LXR activity at LXR response elements (LXREs). We tested whether sterol bound ORP1S stimulates trans-activation activity of LXR using a luciferase trans-activation assay with promoter and enhancer elements of the apoE gene (Fig 9). LXREs are present in the apoE promoter as well as in two enhancer elements (ME.1 and ME.2). ApoE expression is also promoted by a

non-LXRE containing enhancer element (hepatocyte control region 1, HCR1) [31, 38]. We made luciferase expression constructs driven by the *apoE* promoter alone or in tandem with ME.1, ME.2, HCR1, or an inactive ME.1 (ME.1mut) (Fig 9A). These constructs were co-transfected with ORP1S-Myc into HEK-293 cells, which lack detectable endogenous ORP1S expression, and tested for their response to 24S-HC (Fig 9B). ORP1S-Myc and 24S-HC individually or in combination had little effect on transcription from the *apoE* promoter alone as measured by luciferase activity (lanes 1-4); however, the presence of either ME.1 (lanes 5-8) or ME.2 (lanes 9-12) caused a marked increase in the luciferase expression, when both ORP1S and 24S-HC were present (lanes 8 and 12). Neither HCR1 nor the ME.1mut could substitute for ME.1 or ME.2 (lanes 13-20). The inability of the *apoE* promoter alone to respond to ORP1S and 24S-HC suggested that ORP1 and 24S-HC were acting primarily through the ME promoter elements. To test this possibility, the *apoE* promoter was replaced with the heterologous thymidine kinase (TK) promoter (Fig 9C). As with the *apoE* reporter constructs, the tk promoter constructs bearing the ME.1 and ME.2 enhancers, but not the HCR1 or ME.1mut elements promoted luciferase expression in an ORP1S-Myc and 24S-HC dependent manner (Fig 9D), suggesting that LXREs in ME.1 and ME.2 are solely responsible for the stimulation. To determine whether sterol binding to ORP1S is required for LXRE-driven transcription, we compared ORP1S-Myc and ORP1S-P114L-Myc for their ability to promote luciferase expression. ORP1S-P114L does not bind sterol (Fig 1) and the P114L variant did not stimulate luciferase expression regardless of whether 24S-HC was present or not (Fig 9E). These observations suggest that sterol binding to ORP1S is required for LXRE-driven trans-activation activity through the ME.1 and ME.2 enhancers. To test whether ORP1S/24S-HC stabilizes LXR association with LXREs, we mixed purified LXR α -HA and purified RXR α with biotinylated oligos containing the LXRE of ME.1 in the presence or absence of purified GST-ORP1S or GST-ORP1S-P114L that had been either preloaded or not with 24S-HC. Precipitation of the biotinylated oligos showed that wild type GST-ORP1S preloaded with 24S-HC stabilized the association of LXR α with the ME.1 oligo (Fig 9F). These observations indicate that ORP1S facilitates the LXRE-driven trans-activation and suggest that ORP1S may function as a sterol carrier for LXRs.

Discussion

Here, we show that ORP1S translocates from the cytoplasm to the nucleoplasm in response to sterol binding. ORP1S interacts with LXR α/β and promotes oxysterol/LXR-dependent trans-activation of transcription. Because ORP1S and LXR α/β share sterol binding preferences and ORP1S expression is restricted relative to ORP1L [18, 19], we propose that ORP1S functions as a sterol transport protein for the LXR/RXR complex.

Most members of the ORP family have phospholipid and protein interaction modules in their N-terminal domains, which target them to specific cellular membranes where they facilitate exchange of sterols between cellular membrane compartments [1]. Examples include ORP2 and ORP9L. ORP2 has an FFAT motif and transports cholesterol between the plasma membrane, ER and lipid droplets [7]. ORP9L has both an FFAT motif and a PH domain and maintains cholesterol balance within the ER/Golgi system [8]. In some cases, sterol binding promotes membrane interaction, resulting in changes in organelle function. The best characterized example is OSBP, which localizes to Golgi upon 25-HC treatment where it drives sterol-dependent activation of ceramide transport protein (CERT) activity [39] and sphingomyelin synthesis [40]. ORP1S is unusual in that it lacks membrane-targeting motifs and data presented here shows that sterol binding does not target ORP1S to cellular membranes, but rather facilitates translocation of ORP1S from the cytoplasm to the nucleoplasm (Fig 2).

Nuclear import occurs through nuclear pore complexes (NPCs). Molecules less than 40 kDa in size can enter by passive diffusion; however, molecules larger than 40 kDa require active processes [41, 42]. These active processes involve interaction of nuclear localization sequences (NLSs) with the nuclear import machinery. Three types of NLSs have been identified: classical NLS (KKKRK, [43]), “bipartite” NLSs consisting of two shorter clusters of basic amino acids [44] and non-canonical NLSs [45]. Both endogenous ORP1S and the tagged ORP1S variants used in this study exceed the passive diffusion limit and nuclear import of ORP1S required active import (Fig 6) involving both the ¹⁸KKHR²¹ and ²⁵⁰KSKKK²⁵⁴ sequences (Fig 7). Mutation of either sequence abolished the sterol-induced nuclear import of ORP1S without influencing sterol-binding activity. Intriguingly, KKHR is located in a region corresponding to the “lid” region in the crystal structure of the yeast ORP, Osh4p [9]. In Osh4p, sterol binding induces a conformational change in the lid from a poorly-ordered flexible structure to a well-ordered “closed” state [46]. Sterol binding appears to be required for the activity of the NLSs because the binding defective ORP1S-P114L-Myc failed to enter the nucleus in response to cellular cholesterol (Fig 3C) and little nuclear entry occurred with ORP1S-Myc in the absence of sterols (Fig 3A). Sterol binding to ORP1S may induce a conformational change that facilitates exposure of the KKHR sequence to the nuclear transport machinery, a feature that is analogous to ligand-induced nuclear import of the retinoic acid binding protein II [46].

Nuclear transport of oxysterols is unlikely to be a general function of ORP proteins. Most ORP family members lack nuclear localization sequences and have lipid and protein binding modules that anchor them to specific membrane compartments [2, 5, 8, 14, 16, 18, 47, 48]. The one possible exception is ORP2, which has nuclear localization sequences with homology to those of ORP1S. While it is not known whether ORP2 can participate in nuclear sterol transport, ORP2 is normally localized to lipid droplets where it influences triglyceride and cholesterol ester storage and hydrolysis [7, 15]. ORP2 may transport sterols from lipid droplets to the nucleus, while ORP1S may transport sterols through the cytosol to the nucleoplasm.

The binding preference of ORP1S for oxysterols that activate LXR α/β suggested that ORP1S participates in LXR trans-activation. LXR / belongs to a family of nuclear hormone receptors [49], which are activated by small lipophilic molecules: oxysterols for LXR / , retinoids for RAR α and fatty acids for PPAR $\beta/\delta/\gamma$. In general, these lipophilic molecules are transported within cells by both vesicular and non-vesicular mechanisms [50, 51]. Since LXR / and other family members are constitutively located in the nucleus, non-vesicular mechanisms play a major role in the transport of these lipophilic ligands to their cognate nuclear receptors. Non-vesicular transport systems have been identified for delivery of retinoids and fatty acids to RAR and PPAR proteins [28, 29]. These lipophilic ligands are carried by intracellular lipid binding proteins (iLBPs) that bind either retinoids (CRABP-II) or fatty acids (FABP5 and FABP4). After binding, iLBPs migrate to the nucleus and deliver their ligand to RAR α , PPAR β/δ , and PPAR γ , respectively. iLBPs physically interacts with their cognate receptors and transfer ligand, thereby augmenting expression of target genes. ORP1S may act as an iLBP for LXR / . Support for this possibility comes from three observations. First, ORP1S shuttles between the cytoplasm and the nucleoplasm in a sterol dependent manner (Fig 3). Second, sterol species that most efficiently drive ORP1S into the nucleus are the same oxysterols that most potently activate LXR / (Fig 4). Fatty acids, steroids and sterols that do not activate LXR do not drive ORP1S into the nucleus. Lastly, ORP1S binds to LXR and LXR (Fig 8), improves LXR binding to LXREs (Fig 9F) and promotes transcriptional activation by LXRs (Fig 9B/D).

The role of ORP1S in LXR-dependent transcription appears to be restricted to specific LXRE sites. Previous work did not observe ORP1S-dependent augmentation transcription

using a heterologous consensus LXRE-driven luciferase reporter assay [18] and we did not observe ORP1S-dependent augmentation of transcription from the LXRE-containing promoters of apoE (Fig 9) or ABCA1 (data not shown). Instead, ORP1S specifically augmented LXR-dependent transcription via the LXREs in the ME.1 and ME.2 enhancer elements of the apoE gene (Fig 9). The ME.1 and ME.2 enhancer elements play key roles in apoE expression in the brain, but not in liver, where apoE expression depends on the HCR1 enhancer element [52, 53]. ORP1 has highest expression in brain [19], where ORP1S may facilitate apoE production.

In the mature brain, astrocytes produce much of the cholesterol used by neurons [54]. Astrocytes transport cholesterol to neurons via HDL-like lipoproteins that contain apoE. Neurons take up lipoprotein in part through the LDLR, which binds to and internalizes apoE-containing lipoproteins [55]. Neurons promote lipoprotein synthesis in astrocytes through production of oxysterols, which are pumped out of neurons by ABCG1/G4 and taken up by astrocytes where they activate LXR target genes [56, 57]. LXR induces apoE expression via the ME.1/ME.2 elements [53]. Astrocytes express much higher levels of ORP1S than neurons [58] and our finding that ORP1S augments the ability of LXR to activate transcription through the ME elements (Fig 9) suggests that ORP1S may facilitate apoE production in astrocytes, thereby promoting cholesterol transport to neurons.

While it is not fully understood which oxysterols are responsible for in vivo LXR activation in brain [59], alterations in oxysterol production are associated with human disease. For example, Alzheimer's disease is associated with decreased production of 24S-HC and increased production of 27-HC [60]. 27-HC can activate LXR [61]; however, this sterol is poorly transported by ORP1S as compared to 24S-HC (Fig 4). This difference may make LXR less sensitive to 27-HC than to 24S-HC, leading to decreased cholesterol flux from astrocytes to neurons. Decreased cholesterol flux reduces neuronal long term potentiation (LTP), which impairs learning and memory [62]. Of future interest will be testing whether ORP1S is necessary for cholesterol transport from astrocytes to neurons and whether loss of ORP1S function compromises LTP.

In conclusion, our findings show that ORP1S binds to cholesterol and specific oxysterols. This interaction allows ORP1S to translocate from the cytoplasm to the nucleoplasm, where ORP1S facilitates the ability of LXR to bind to LXREs and activate transcription. This ability may play a role in cholesterol transport between astrocytes and neurons.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- ORP1S translocates to the nucleus in response to sterol binding.
- The sterols that best promote nuclear import of ORP1S are LXR agonists.
- ORP1S binds to LXRs, enhances binding of LXRs to LXREs and promotes LXR-dependent transcription of ApoE.

A Domain Structure of ORP1 Subfamily and OSBP

B Sequence alignment of ORPs near P114 of ORP1S

Human	ORP1S	101	VASQWERT-G-KPFN P LLGETY	120	Human	ORP1S	101	VASQWERT-G-----KPFN P LLGETY	120
(Hs)	ORP1L	584	VASQWERT-G-KPFN P LLGETY	633	Yeast	Osh1p	858	AAFTASMY-ASTTNRVSKPFN P LLGETF	884
	ORP2	99	VASQWERT-G-KPFN P LLGETY	148	(Sc)	Osh2p	956	AAFTASSY-ASTTKRVAKPFN P LLGETF	982
	ORP3	563	YASSYYRA-GSKPFN P VLGETY	613		Osh3p	701	AISFLSIY-RDKTRTLRKPFN P LLAETF	727
	ORP5	407	FYKKPKGI--KKPN P ILGETF	459		Osh4p	93	LKSQYCSR-NESLGSEKKPLN P FLGELF	119
	ORP6	606	YCSTYFRA-GSKPFN P VLGETY	656		Osh5p	93	LRSQYCSR-SESMGSEKKPLN P FLGEVF	119
	ORP7	514	YSSTYHRA-GCKPFN P VLGETY	564		Osh6p	110	MKWYLAGW-HIAPKAVKKPLN P VLGEYF	136
	ORP8	443	FYKKPKGL--KKPN P ILGETF	492		Osh7p	110	VAWYLAGW-HIGPRAVKKPLN P ILGEHF	136
	ORP9	411	FHAGRKGSVAKKPYN P ILGEIF	462					
	ORP10	436	FHEGRKGALAKKPYN P IIGETF	487	Worm	OBR-1	447	YSTTCHRTN-----KPFN P LLGETF	466
	ORP11	406	FHEGRKGAIKKPYN P IIGETF	453	(Ce)	OBR-2	110	VSANHKRL-----SKPFN P LLLETF	119
	OSBP	454	YSTTVFRT--SKPFN P LLGETF	503		OBR-3	374	FYKKPKGL-----KKPN P ILGETF	393
	OSBP2	558	YSTTVHRI-A-KPFN P MLGETF	607		OBR-4	384	AARKSGVA-----KKPYNA P ILGETF	403

Hs : *Homo Sapiens*

Sc : *Saccharomyces cerevisiae*

Ce : *Caenorhabditis elegans*

Figure 1. Domain structures of ORP1 subfamily and sequence alignment of conserved regions near P114L of ORP1S

A) Domain structures of OSBP, ORP1L, and ORP1S are shown. **B)** Sequences of ORP paralogs and the yeast and *C. elegans* homologs near P114 of ORP1S are shown.

Figure 2. Sterol determines nuclear location of ORP1S

A-C) For each sterol binding reaction, purified recombinant human GST-ORP1S (●) or GST-ORP1SP114L (■) proteins (50 pmol) on agarose beads was incubated in the presence of the indicated concentration of [3 H]-cholesterol (**A**), [3 H]-25-hydroxycholesterol (25-HC) (**B**) or [3 H]-22R-HC (**C**). **D-F)** For each competitive binding reaction, the indicated concentration of cholesterol (CL) (**D**), 25-HC (**E**), or 22R-HC (**F**) was incubated with 10 nM of [3 H]-labeled sterol and 10 pmol of purified GST- ORP1S (●) immobilized on beads. Beads were washed and bound [3 H]-cholesterol (**A, D**), [3 H]-25-HC (**B, E**) or [3 H]-22R-HC (**C, F**) were determined by scintillation counting. Specific binding was calculated by

subtracting radioactivity precipitated by 10 pmol of purified GST immobilized on beads from the radioactivity precipitated by GST-ORP1S. The graphs shown are representative of three independent experiments. The estimated binding affinity (**A-C**) is expressed in $K_d \pm SD$. The binding affinity of ORP1S P114L for sterols was too weak to be estimated in our assay system. **G,H**) HeLa cells were transfected with cDNAs encoding C-terminal Myc-tagged ORP1S or ORP1SP114L and grown for 17 hr in normal growth medium (medium A). The cells were fixed and processed for the localization of Myc-tagged proteins, as described in the experimental procedures. N = Nucleus, Bar, 10 μm .

Figure 3. Cellular cholesterol regulates nuclear import of ORP1S

HeLa cells were transfected with cDNAs encoding C-terminal Myc tagged ORP1S (A), C-terminal GFP tagged ORP1S (B/E), C-terminal Myc tagged ORP1S P114L (C) or C-terminal V5 tagged ORP1L (D). Cells were cultured in either normal growth medium (medium A, columns 1 and 2) or in cholesterol depletion medium (medium B, which contains 5% lipoprotein poor serum, 50 μM compactin and 50 μM mevalonate, columns 3-5) for 12 hr. Medium A cultured cells were either processed for immunofluorescence directly (10% FBS) or processed following treatment with 0.5% methyl β-cyclodextrin in medium B for 1 hr (+0.5 % MCD). Cells cultured overnight with medium B were either processed directly (5% LPPS + C/M) or processed following a 2 hr incubation with cholesterol (CL) or epicholesterol (Epi) (20 μM). Myc, GFP, V5 and STAT3 immunostaining were visualized by confocal fluorescence microscopy. Bar, 10 μm.

Figure 4. Specific oxysterols stimulate nuclear import of ORP1S-GFP

HeLa cells transfected with ORP1S-GFP and cultured in medium B for 12 hr, were incubated in the presence of either medium B alone (LPPS) or medium B containing 10 μ M of indicated ligand for 2 hr. Cells were then fixed and processed for confocal immunofluorescence microscopy. Bar, 10 μ m.

Figure 5. Oxysterols that potently stimulate nuclear import of ORP1S-GFP bind strongly to ORP1S

A, B) The nuclear and cytoplasmic fluorescence intensities of HeLa cells expressing ORP1S-GFP, cultured in medium B and treated with indicated concentration of sterol, were quantified from confocal images of 50 cells using ImageJ and plotted as the average ratio of nuclear to cytoplasmic fluorescence intensity (N/C). Error bars indicate $\pm$ SD. The graphs shown are representative of three independent experiments. **C, D)** Purified GST-ORP1S (50 pmol) immobilized on beads was incubated in the presence of 10 nM [3 H]-25-HC and the indicated concentration of unlabeled 22R-HC (●), 22S-HC (○) or 24S-HC (■) (D) or 24,25-epoxycholesterol (■), 20S-HC (Δ) or 25-HC (◇) (E). Non-specific binding was assessed in parallel assays using purified GST immobilized on beads and subtracted from total binding to generate specific binding. Specific binding is presented as a percentage of [3 H]-25-HC in the absence of competing sterol. Data presented is the average of three independent experiments. Error bars indicate $\pm$ SD.

Figure 6. ORP1S-GFP is actively imported into the nucleus

A) HeLa cells expressing photoactivatable GFP (PAGFP) or ORP1S-PAGFP were cultured overnight in medium B and either photoactivated directly (LPPS) or photoactivated following incubation with 10 μ M 22R-HC in medium B (22R) for 2 hr. Time-lapse images of HeLa cells were taken once before activation, immediately after activation and every 5 sec for 5 min thereafter. The images at indicated time are shown (min:sec). Before indicates before photoactivation. Activation indicates the images immediately following photoactivation. The open circle indicates the activated area. **B)** Quantification of fluorescence intensity of the cytosol (O) and the nucleus (●) relative to fluorescence intensity before activation is

plotted as a function of time. The half time ($t^{1/2}$) necessary for nuclear fluorescence to reach equilibrium was determined using nonlinear regression analysis.

Figure 7. Nuclear Localization Signal sequence of ORP1S

A) Putative nuclear localization signal (NLS) sequences in the primary structure of ORP1S, the mutations made to these sequences, the designations of the variants and the relative ability of each variant to enter the nucleus in response to 22R-HC are shown. **B)** HeLa cells transfected with cDNAs encoding C-terminal Myc-tagged wild type or NLS mutants of ORP1S were cultured in medium A (FBS), cultured in medium B (LPPS), or cultured in medium B and treated with 10 μ M 22R-HC for 2 hr (22R). Cells were then fixed and processed for immunofluorescence using anti-Myc antibody. **C)** 10 nM [3 H]-22R-HC was incubated with 10 pmol of GST, GST-ORP1S or GST-K18 bound to agarose in the presence or absence of increasing concentrations of unlabeled 22R-HC. Binding to GST alone beads was subtracted from GST-ORP1S and GST-K18 data to generate specific binding. Data is plotted as a percentage of binding in the absence of unlabeled 22R-HC for both wild type

(●) and K18 (O) mutant. Presented data is the average of three independent determinations. Error bars indicate $\pm$ SD.

Figure 8. ORP1S interacts with LXR /

A) Purified GST or GST-ORP1S (50 pmol) on agarose beads was incubated with nuclear extracts from HEK293 cells stably overexpressing LXR α , LXR β or neither that had been treated with 24S-HC (10 μ M, **A**) for 2 hr at room temperature. The beads were washed four times, eluted with SDS sample buffer, run on SDS-PAGE gels and either immunoblotted with α -HA mAb or stained with Coomassie Blue. **B, C)** HEK293T cells were transfected with the indicated plasmids. GFP or GFP fusion proteins (**B**) or HA fusion proteins (**C**) were immunoprecipitated from nuclear extracts of transfected cells. The precipitates (lanes 1-3), supernatant (lanes 4-6) and a fraction of the nuclear extract input (lanes 7-9) were analyzed by immunoblotting with α -HA mAb (**B**) or α -GFP mAb (**C**). Arrowheads indicate the bands corresponding to LXR -HA, ORP1S-GFP and GFP. Normal rabbit Ab was used as a negative control for the HA immunoprecipitations (**C**). The input loaded on the gels was 2 % of the material used in the immunoprecipitations.

Figure 9. OPR1S drives LXRE-dependent transactivation by ME.1 and ME.2 and facilitates LXR binding to LXREs

A/C) Structural elements of promoter constructs containing the apoE proximal promoter (**A**) or the tk promoter (**C**) and the indicated enhancers in pGL-Luciferase plasmid constructs are diagrammed. The numbering of apoE promoter was based on the transcription start site (+1) of ApoE gene. **B,D)** HEK293 cells were transfected with the indicated firefly luciferase plasmids, pCMX-RXRα, pCMX-LXRα and pRL-tk. Indicated cells were also co-transfected with ORP1S-Myc. Cells were then incubated for 24 hr in medium B followed by 2 hr treatment with 10 μM 24S-HC (+) or vehicle control (-). Firefly luciferase activity was normalized for transfection efficiency using Renilla luciferase activity derived from the pRL-tk plasmid. **E)** HEK293 cells were transfected with the indicated firefly luciferase plasmids, pCMX-RXRα, pCMX-LXRα and pRL-tk. Indicated cells were also co-transfected with ORP1S-Myc or ORP1S-P114L-Myc. Cells were then incubated for 24 hr in medium B followed by 2 hr treatment with 10 μM 24S-HC (+) or vehicle control (-). Firefly luciferase activity was normalized for transfection efficiency using Renilla luciferase activity derived from the pRL-tk plasmid. All data shown is the average of four independent experiments. Error bars indicate ±SD. **F)** Biotin-labeled DNA oligonucleotides bearing the LXRE

sequence of wild type ME.1 (ME.1) or mutated ME.1 (ME.1mut) was pre-loaded on streptavidin agarose beads and incubated with purified LXR α -HA, RXR α and GST-ORP1S (wt) or GST-ORP1S-P114L (PL). GST-ORP1S and GST-ORP1S-P114L were preloaded with 22S-HC by incubating the GST-ORP1S fusions with 10 μ M 22S-HC, loading on glutathione agarose, washing and eluting with glutathione. DNA-bound proteins were pulled down and analyzed by immunoblotting for HA and RXR α . A-C, *, $P<0.05$, **, $P<0.01$, ***, $P<0.001$.