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Research paper

ORP2 interacts with phosphoinositides and controls the subcellular distribution of cholesterol

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ABSTRACT

ORP2 is a sterol-binding protein with documented functions in lipid and glucose metabolism, Akt signaling, steroidogenesis, cell adhesion, migration and proliferation.

Here we investigate the interactions of ORP2 with phosphoinositides (PIPs) by surface plasmon resonance (SPR), its affinity for cholesterol with a pull-down assay, and its capacity to transfer sterol *in vitro*. Moreover, we determine the effects of wild-type (wt) ORP2 and a mutant with attenuated PIP binding, ORP2(mHHK), on the subcellular distribution of cholesterol, and analyze the interaction of ORP2 with the related cholesterol transporter ORP1L.

ORP2 showed specific affinity for PI(4,5)P₂, PI(3,4,5)P₃ and PI(4)P, with suggestive K_d values in the μM range. Also binding of cholesterol by ORP2 was detectable, but a K_d could not be determined. Wt ORP2 was in HeLa cells mainly detected in the cytosol, ER, late endosomes, and occasionally on lipid droplets (LDs), while ORP2(mHHK) displayed an enhanced LD localization. Overexpression of wt ORP2 shifted the D4H cholesterol probe away from endosomes, while ORP2(mHHK) caused endosomal accumulation of the probe. Although ORP2 failed to transfer dehydroergosterol in an *in vitro* assay where OSBP is active, its knock-down resulted in the accumulation of cholesterol in late endocytic compartments, as detected by both D4H and filipin probes. Interestingly, ORP2 was shown to interact and partially co-localize on late endosomes with ORP1L, a cholesterol transporter/sensor at ER-late endosome junctions.

Our data demonstrates that ORP2 binds several phosphoinositides, both PI(4)P and multiply phosphorylated species. ORP2 regulates the subcellular distribution of cholesterol dependent on its PIP-binding capacity. The interaction of ORP2 with ORP1L suggests a concerted action of the two ORPs.

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Abbreviations: BiFC, bimolecular fluorescence complementation; DHE, dehydroergosterol; ER, endoplasmic reticulum; GST, glutathione-S-transferase; K_d, dissociation constant; LD, lipid droplet; LE, late endosome; OHC, hydroxysterol; ORD, OSBP-related domain; ORP, OSBP-related protein; OSBP, oxysterol-binding protein; PIP, phosphoinositide; PI(4)P, phosphatidylinositol-4-phosphate; PI(4,5)P₂, phosphatidylinositol-4,5-bisphosphate; PI(3,4,5)P₃, phosphatidylinositol-3,4,5-trisphosphate; PM, plasma membrane; SPR, surface plasmon resonance; VAPA, VAMP-associated protein A.

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1. Introduction

Phosphatidylinositol phosphates (PIPs) are phosphorylated derivatives of phosphatidylinositol (PI), in which the inositol ring head group is phosphorylated at positions of 3, 4 and/or 5, resulting in seven different PIPs. These lipids are of low abundance in cells and are mostly known for their signaling functions, playing central roles e.g. in membrane trafficking, cell migration and signal transduction [1]. The distribution of the distinct PIP species varies between different cellular membranes, as they are generated through the action of kinases with distinct subcellular localizations and turned over by specific phosphatases. For example, PI(4)P localizes

to the Golgi apparatus and the plasma membrane (PM), PI(4,5)P₂ and PI(3,4,5)P₃ to the PM and endosomes, PI(3,5)P₂ to late endosomes and PI(3)P to early endosomes [1,2]. The recruitment of many proteins to membranes occurs via specific PIP-binding domains with different affinities for the distinct PIP species.

OSBP-related proteins constitute a ubiquitously expressed family of 12 mammalian proteins [3–5]. The ORP proteins are characterized by a lipid-binding domain designated ORD (OSBP-related domain) in their carboxy-terminal half. The ORD of several ORPs binds cholesterol, oxysterols, or phosphatidylserine (PS), and PI(4)P or other PIPs [6–12]. In addition, most ORPs carry an amino-terminal region with a membrane-targeting pleckstrin homology (PH) domain and a two phenylalanines in an acidic tract (FFAT) motif that interacts with the integral VAMP-associated proteins (VAPs) of the ER [13,14]. Several ORPs are shown to localize at membrane contact sites (MCSs), zones of close apposition of two organelle limiting membranes [15]. Such sites are known to mediate the spatially restricted and tightly regulated inter-organelle transfer of lipids, Ca²⁺ ions, and other signals [16,17]. Recent hallmark studies have established that a number of ORPs have the capacity to mediate the counter-current transport of cholesterol or phosphatidylserine (PS) in exchange for PIPs, a process in which the synthesis and hydrolysis of PIPs energizes the transport of cholesterol or PS against their concentration gradients [6,10,11,18].

ORP2 is the only mammalian ORP protein family member that exists solely as a 'short' subtype lacking a PH domain [3–5]. However, ORP2 was in certain cell lines found to target the surface of cytoplasmic lipid droplets (LD), and via interaction with the VAPs, ORP2 localizes to MCSs between the ER and LDs [19,20]. In addition, ORP2 is found in the cell cortex or at the PM [19,20] (Fig. 1). Our recently published observations revealed novel functions of ORP2

in actin cytoskeletal regulation, Akt signalling, cellular energy metabolism, adhesion, migration and proliferation [21,22]. The actin regulatory effect of ORP2 appeared to depend on the ability of ORP2 to bind PIPs, as a PIP-binding defective mutant of ORP2 failed to induce the alterations of F-actin organization that the wild-type protein caused, and was unable to rescue a migration defect observed in ORP2 knock-out hepatocytes [21].

Of note, ORP2 is also suggested to play a role in adrenocortical steroidogenesis [23] and cellular cholesterol homeostasis [23–25]. ORP2 overexpression was reported to reduce the cellular free cholesterol by enhancing cholesterol efflux [24,25]. Furthermore, double knock-down of ORP2 and the closely related ORP1S impeded cholesterol transport from the PM to the ER [26].

A number of reports have addressed the lipid ligands of ORP2 by employing charcoal-dextran, pull-down or lipid-protein overlay assays (Table 1). The protein is shown to bind sterols [19,27], PIPs on vesicles [24] and showed affinity for phosphatidic acid and cardiolipin in an overlay assay [28]. However, previous work has not documented the affinity of ORP2 for PIPs and cholesterol, nor has its function as a putative intracellular transporter of cholesterol been addressed in detail. In this study we investigate the interactions of ORP2 with PI(4)P, PI(4,5)P₂, and PI(3,4,5)P₃ by surface plasmon resonance (SPR) analysis, as well as its affinity for cholesterol with a pull-down assay. The ability of ORP2 to transport dehydroergosterol (DHE) *in vitro* is assayed. Moreover, we employ fluorescent probes to determine the effects of ORP2 and its mutant with attenuated PIP binding on the subcellular distribution of cholesterol, and analyze the interaction of ORP2 with the related ORP1L.

2. Materials and methods

2.1. Antibodies and reagents

Anti-Xpress[®] antibody was purchased from Invitrogen/Thermo Scientific (Carlsbad, CA), anti-GFP antibody from Molecular Probes/Thermo Scientific (Eugene, OR), anti-Rab7 antibody from Santa Cruz Biotechnology (Dallas, TX) and anti-LAMP1 monoclonal antibody (clone H4A3) from the Developmental Studies Hybridoma Bank (Iowa City, IA). Alexa Fluor647-dextran, BODIPY 493/503 and

Fig. 1. A schematic image on the subcellular localizations of ORP2. ORP2 is in principle a cytosolic protein that associates with the ER via binding to the VAP proteins [25,29]. (A) In cells with prominent lipid droplets (LDs) ORP2 is often seen encircling the LDs [19]. When coexpressing ORP2 with the endoplasmic reticulum (ER) VAP proteins, the ORP2-VAP complexes localize at ER-LD contacts [20,29]. (B) In motile cells ORP2 is found in the cell cortex and co-localizes with the dynamic F-actin at the leading edge [21]. (C) In the present study we demonstrate that ORP2 also localizes on late endosomal/lysosomal compartments (LE/Lys). The non-LD localizations of ORP2 become obvious in cells that do not have large LDs. (D) ORP2 overexpression enhances cholesterol efflux [24,25]; FC, free cholesterol.

Table 1
A summary of ORP2 ligand information.

Ligand	K _D	Method	Reference
22(R)OHC ^a	1.4 × 10 ⁻⁸ M	Charcoal-dextran assay	[19]
7-KC ^b	1.6 × 10 ⁻⁷ M	Charcoal-dextran assay	[19]
25-OHC	3.9 × 10 ⁻⁶ M	Charcoal-dextran assay	[27]
Cholesterol	ND ^c	Pull-down assay	[19]; This study
PI(3,4,5)P ₃ ^d	ND	Pull-down of vesicles	[24]
	76 × 10 ⁻⁶ M ^e	SPR ^f	This study
PI(3,4)P ₂	ND	Pull-down of vesicles	[24]
	ND	Lipid-protein overlay	[28]
PI(3,5)P ₂	ND	Pull-down of vesicles	[24]
PI(4,5)P ₂	ND	Pull-down of vesicles	[24]
	ND	Lipid-protein overlay	[28]
	52 × 10 ⁻⁶ M ^e	SPR	This study
PI(4)P	305 × 10 ⁻⁶ M ^e	SPR	This study
PI(3)P	ND	Lipid-protein overlay	[28]
Phosphatidic acid	ND	Lipid-protein overlay	[28]
Cardiolipin	ND	Lipid-protein overlay	[28]

^a Hydroxycholesterol.

^b Ketcholesterol.

^c Not determined.

^d Phosphatidylinositol-3,4,5-trisphosphate.

^e The K_d values must be considered suggestive, due to substantial residual binding by ORP2(mHHK), a triple point mutant of the inositol-phosphate binding cleft.

^f Surface plasmon resonance.

BODIPY 558/568 C₁₂ neutral lipid dyes were from Molecular Probes. The Switchavidin™ used for SPR measurements was from BioNavis (Tampere, Finland) and the diC8 phosphoinositides from Echelon Biosciences (Salt Lake City, UT). TALON®-resin was from Clontech/Takara Bio (Mountain View, CA), the Strep-Tactin® resin and D-Des-thiobiotin from IBA Lifesciences (Goettingen, Germany), and Protein G Magnetic beads from Pierce/Thermo Scientific (Waltham, MA). [1,2-³H(N)]cholesterol was from PerkinElmer (Waltham, MA), egg phosphatidylcholine (PC), egg phosphatidylethanolamine (PE), porcine brain phosphatidylserine (PS), diC18:1-PI(4)P, diC18:1-PI(4,5)P₂, dehydroerogosterol (DHE), 1,2-dioleoyl-*sn*-glycero-3-[(N-(5-amino-1-carboxypentyl)iminodiacetic acid) succinyl](DGS-NINTA) and 18:1-dansyl-phosphatidylethanolamine from Avanti Polar Lipids (Alabaster, AL). The Silencer Select® OSBPL2 and OSBPL1A siRNAs and the Negative Control #2 siRNA were from Ambion/Thermo Scientific (Austin, TX), and Niemann-Pick C1 (NPC1)-specific siRNA targeting the sequence CCAGGTTCTTGACTTACAA from Sigma-Aldrich (St. Louis, MO). Filipin (F9765) was purchased from Sigma-Aldrich (St. Louis, MO).

2.2. cDNA constructs

Human ORP2 wild-type, phosphoinositide-binding attenuated (mHHK; H^{178–179}A, K⁴²³A) and VAP-binding defective (mFFAT; F^{7–8}V, D⁹V) mutants in pmCherry-C1 (Clontech/Takara Bio) were described in Ref. [29], and GFP-tagged versions of these mutants were generated by transferring the cDNAs into the BglIII/KpnI restriction sites of pEGFP-C1 (Clontech/Takara Bio). Constructs for rescue of D4H distribution after knock-down of ORP2 or ORP1L were created by generating three silent mutations in the siRNA target sequences of the corresponding cDNAs, by using QuikChange II XL mutagenesis kit (Agilent, Santa Clara, CA). The ORP2 expression construct in pcDNA4HisMax-C (Invitrogen/Thermo Scientific, Carlsbad, CA) was described in Ref. [19]. The mCherry-D4H cholesterol probe plasmid was a kind gift from Prof. Gregory Fairn (Department of Biochemistry, University of Toronto, ON).

pFOLD-1 is a novel bacterial expression vector that enhances the solubility of mammalian proteins in *E. coli*. This vector is based on gene 9 of the T7 phage (T7 scaffolding protein) that was cloned into the pBAT4 vector [30]. Open reading frames to be expressed are fused to the C-terminus of the T7 scaffolding protein and the fusion proteins are then expressed from a T7 promoter. Shortly, the vector consists of the T7 promoter, the T7 gene 9, a His₆ or Strep tag sequence, a thrombin cleavage site, and a multiple cloning site. For production of ORP2 in *E. coli*, the wild-type and ORP2(mHHK) cDNAs were cloned into the BamHI site of pFOLD-His₆ and pFOLD-Strep. For production of ORP2 with two Strep tags at its carboxy-terminus (ORP2-2xStrep) in insect cells, the cDNA was engineered and cloned into pFastBac1 (Thermo Fisher Scientific, Waltham, MA). The pEGFP-C1-ORP1L construct is described in Ref. [31].

For Bimolecular fluorescence complementation, ORP2 cDNA was inserted into the BglIII/KpnI sites of pmVN-C encoding aa 1–172 of Venus, and ORP1L cDNA into the corresponding sites in pmVC-C encoding aa 155–238 of Venus [32].

2.3. Expression and purification of ORP2 and VAPA recombinant proteins

For recombinant protein purification, ORP2 proteins from the pFOLD-Strep or pFOLD-His₆ vectors were produced in *E. coli* Rosetta 2 (DE3) (Novagen/Merck, Darmstadt, Germany). Expression was induced with 0.5 mM IPTG at 18 °C for 24 h. Cells were harvested and resuspended in 100 mM Tris–HCl (pH 8.0), 150 mM NaCl, complete Protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany) and lysed by sonication. Lysates were centrifuged

(15 min 20 000×g, 4 °C), and the N-terminally pFOLD-Strep-tagged ORP2 proteins were purified from the supernatant using Strep-Tactin resin (IBA Lifesciences), and eluted with 2.5 mM D-Des-thiobiotin (IBA Lifesciences), 100 mM Tris–HCl (pH 8.0) and 150 mM NaCl. The pFOLD-His₆-tagged ORP2 was purified using Ni-NTA agarose (Invitrogen, Carlsbad, CA) and eluted with 0.5 M imidazole. The control proteins produced from the corresponding empty vectors were expressed and purified identically. ORP2-2xStrep was produced with a baculovirus vector in *Spodoptera frugiperda* Sf9 cells at BioMediTech Protein Technologies Core Facility (University of Tampere and the Tampere University of Technology, Tampere, Finland) and purified on the Strep-Tactin resin according to the manufacturer's instructions. The cytosolic domain of human VAPA with a carboxy-terminal His₆ tag was produced in *E. coli* Rosetta 2 and purified on Ni-NTA Agarose (Invitrogen) with a standard protocol. Coomassie stained gels of the recombinant proteins are displayed in Supplemental Fig. S1.

2.4. Cell culture, transfection and RNA interference

Human HeLa cells were cultured in high glucose Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich) supplemented with 10% FBS, 4 mM L-glutamine, 100 µg/ml streptomycin and 100 U/ml penicillin (Sigma-Aldrich). HuH7 hepatoma cells were cultured as described in Ref. [19]. Lipofectamine 2000® (Invitrogen, Carlsbad, CA) or JetPRIME® (Polyplus, New York City, NY) were used for cDNA transfections according to the manufacturer's instructions. The OSBPL2, OSBPL1A, NPC1 and control siRNAs were transfected using Oligofectamine™ (Invitrogen) by the manufacturer's protocol. Twenty-four hours after siRNA transfections, plasmids and additional 130 nM siRNAs were transfected with Lipofectamine® 2000 and incubated for another 24 h. For the filipin stainings, siRNA transfections were carried out for 48 h.

2.5. Co-immunoprecipitation

HuH7 cells were transfected with pEGFP-C1-ORP1L and pcDNA4HisMax-C-ORP2 constructs as specified above. Plain pEGFP-C1 and pcDNA4HisMax-C vectors were used as negative controls. After 24 h, cells were washed with PBS and lysed in lysis buffer (10 mM HEPES pH 7.6, 150 mM NaCl, 0.5 mM MgCl₂, 10% glycerol, 0.5% Triton X-100, 0.5% Na-deoxycholate, Protease inhibitor cocktail, Roche Diagnostics), mixed thoroughly by vortexing and incubated on ice for 10 min. Unbroken cells and insoluble material were removed by centrifugation at 20,000×g for 10 min. The obtained lysates were mixed with 30 µl of Protein G Magnetic Beads (Pierce/Thermo Fisher Scientific) and incubated for 30 min on rotation at 4 °C to remove material binding unspecifically to the beads. After removing the beads, 2 µl of anti-Xpress antibody (Thermo Scientific) was added and incubated for 24 h on rotation at 4 °C. The next day, 30 µl of Protein G Magnetic Beads were added and the mixture was incubated for 2 h on rotation at 4 °C. The beads were then washed three times with lysis buffer, resuspended in 45 µl Laemmli sample buffer and boiled for 5 min. The bound proteins were detected with anti-GFP (Thermo Scientific) and anti-Xpress antibodies.

2.6. Cholesterol binding assay

The specific affinity of purified recombinant ORP2 to cholesterol was defined with an assay described previously [33] with minor modifications. Recombinant pFOLD-His₆ was used as a negative control. pFOLD-His₆-ORP2 and the pFOLD-His₆ were incubated at a 70 nM concentration with 20–400 nM mixtures of unlabeled and [³H]cholesterol for 2 h at room temperature in 20 mM Tris (pH 7.4),

100 mM KCl, 0.05% Triton X-100 or 0.1% CHAPS (Amresco, Boise, ID). Subsequently, 20 μ l TALON[®] resin (Takara Bio) was added in each sterol-protein mix and incubated for 30 min at RT with constant rotation. The supernatants were removed and the beads were washed 3 times with the above buffers. The amounts of bound ORP2-cholesterol complex were determined by liquid scintillation counting.

2.7. Dehydroergosterol (DHE) transfer assays

The ability of ORP2 to transfer DHE was assessed essentially as described in Ref. [10]. The assays donor liposomes (L_d) composed of 75 mol% egg-PC, 5 mol% porcine brain PS, 2 mol% 18:1 DGS-NiNTA, 18 mol% DHE, and the acceptors of 73.5 mol% egg-PC, 19 mol% egg PE, 5 mol% porcine brain PS, and 2.5 mol% dansyl-PE. The assay buffer was 50 mM HEPES, pH 7.2, 120 mM K-acetate, 1 mM $MgCl_2$ (HKM buffer). In some assays, 2 mol% di18:1-PI(4)P (L_{a1}) or di18:1-PI(4,5)P₂ (L_{a2}) was included in the acceptor liposomes, with a corresponding reduction in the mol% of egg PC. The assays contained 10 nmol L_d and 10 nmol L_a lipid in a total volume of 150 μ l. VAPA-His₆ (50 pmol per assay) was added to the donor liposomes and incubated for 30 min at room temperature before the assays. The DHE–dansyl-PE FRET signal was recorded at 3-min intervals for 60 min on an EnSpire fluorometer/multimode plate reader (Perkin Elmer) with the excitation and fluorescence detection wavelengths of 310 and 525 nm, respectively. In the transfer assays, 15 pmol (final conc. 100 nM) plain pFOLD-His₆, pFOLD-His₆-ORP2, ORP2-2xStrep, OSBP (a kind gift from Prof. Bruno Antonny, Institut de Pharmacologie Moléculaire et Cellulaire, Valbonne, France) were added per reaction.

2.8. Dynamic light scattering (DLS) measurements

The liposome tethering ability of ORP2 was studied by DLS using a Zetasizer APS instrument (Malvern, UK). The assay was designed as described in Ref. [10], and liposomes identical to those used in the DHE transfer assay (L_d , L_{a1} , L_{a2}) were used. L_d (25 μ M) were pre-incubated with 200 nM of VAPA-His₆ for 30 min at room temperature, mixed with L_{a1} (25 μ M) or L_{a2} (25 μ M) in HKM buffer, following addition of 250 nM of plain pFOLD-Strep, pFOLD-Strep-ORP2, ORP2-2x-Strep or OSBP. The liposome aggregation was measured at 30 °C at 2-min intervals, each time point consisting of 13 autocorrelation runs. The data was collected and analyzed by Malvern software by using cumulate method, i.e. the particle diameter was fitted as intensity and polydispersity index–based Gaussian distribution.

2.9. Preparing surface plasmon resonance (SPR) sensor chip surfaces

All SPR experiments were performed with an MP-SRP NAVI 220A instrument (BioNavis, Tampere, Finland) equipped with 2 flow channels. The binding affinities of ORP2 for PIPs were assessed by using functionalized biotin-coated gold sensors (BioNavis). Prior to the experiments, the sensors were coated with Switchavidin[™] (a neutralized avidin mutant for reversible binding, BioNavis) to create an affinity surface for the strep-tagged recombinant proteins. The Switchavidin was immobilized onto the sensor chip surface according to manufacturer's instructions. Subsequently, pFOLD-Strep-ORP2 or pFOLD-Strep-ORP2(mHHK) (flow channel 1) and pFOLD-Strep as reference (flow channel 2) were allowed to form a complex with the biotin-Switchavidin surface. The proteins were immobilized using a flow rate of 20 μ l/min and a protein concentration of 50 μ g/ml until the binding reached saturation. The unbound proteins were removed by washing the sensor surface

with a 20 mM HEPES, 150 mM NaCl (pH 7.4) running buffer until the SPR signal was stabilized.

2.10. Surface plasmon resonance (SPR) measurements

The binding affinities of diC8-PI(4)P, -PI(4,5)P₂, -PI(3,4,5)P₃ to ORP2 were determined in real-time using an MP-SPR NAVI 220A instrument (BioNavis) which utilizes the Kretschmann configuration to excite surface plasmons. The whole SPR angular spectrum was measured between 60 and 75°, and scanned with a laser wavelength of 670 nm. All measurements were carried out by using a 20 mM HEPES, 150 mM NaCl (pH 7.4) running buffer at 20 °C with a flow rate of 20 μ l/min. The SPR sensorgrams were measured for a series of injections of PIPs (0.33–100 μ M) at growing concentrations. To distinguish specific affinities from possible unspecific binding and the bulk effect, i.e. the refractive index change caused by merely rising PIP or protein concentrations, pFOLD-Strep was used as a control. Samples were injected sequentially through the two parallel SPR flow channels containing pFOLD-Strep-ORP2 or pFOLD-Strep-ORP2(mHHK) (flow channel 1) and pFOLD-Strep as the reference (flow channel 2). The SPR sensorgram from the reference channel was subtracted from the ORP2 channel in real-time. The data was collected with the MP-SPR Navi Control 4.2.5 software (BioNavis) and processed using the MP-SPR Navi Data Viewer 4.2.5 software (BioNavis). The dissociation constants were determined by using the TraceDrawer 1.7 software (RidgeView Instruments AB, Uppsala, Sweden). The quality of the fit was determined with χ^2 value (Supplemental Table S1).

2.11. Fluorescence microscopy

The fluorescent labels were visualized with a Zeiss Axio Observer Z1 microscope equipped with PlanApo x63/1.40 oil DICII objective and a Colibri LED light source (Zeiss, Oberkochen, Germany). Images were taken with a AxioCam HRm camera and recorded with Zen 2 v.2.0.0 software (Zeiss). For live cell imaging, a stage top incubation chamber (Zeiss) with temperature set to 37 °C and CO₂-independent cell culture medium (Gibco/Thermo Scientific) were used. Adobe Photoshop CC 2017 (Adobe, San Jose, CA), Imaris 9.1 (Bitplane, Zürich, Switzerland) and ImageJ/Fiji (National Institutes of Health, Bethesda, MD) were used for image processing and analysis. Colocalization analysis was performed with ImageJ coloc2 function and determined by Pearson's correlation coefficient.

2.12. Bimolecular fluorescence complementation (BiFC)

HeLa cells were double transfected for 24 h with pmVN-C-ORP2 and pmVC-C-ORP1L, or a single BiFC construct was cotransfected with an empty vector as a negative control. In some experiments Alexa Fluor647-dextran was internalized for 90 min and chased for 1 h to label late endosomes/lysosomes, or these organelles were stained with anti-Rab7. LD were stained with BODIPY 558/568 C₁₂. The cells were fixed and processed for fluorescence microscopy as described above.

2.13. Staining of cholesterol with filipin

HeLa cells were transfected for 48 h with control or ORP2 siRNA using HiPerFect (Qiagen, Hilden, Germany). After fixation with 4% paraformaldehyde for 15 min, cells were stained with 0.5 mg/ml filipin in 1% BSA/PBS for 1 h, stained with anti-LAMP1 (clone H4A3) and with secondary antibodies. Cells were imaged with a Nikon (Tokyo, Japan) Eclipse Ti-E inverted microscope equipped with a 40x/0.75 objective. Mean filipin fluorescence intensity per cell and

in LAMP1 positive organelles was quantified using ImageJ/Fiji software, and statistical significance of differences was evaluated with Student's *t*-test.

3. Results

3.1. Binding of phosphoinositides and cholesterol to ORP2

We recently proposed that ORP2 is involved in regulation of adhesion, migration and proliferation of HuH7 hepatoma cells – functions that were reliant on the ability of ORP2 to bind PIPs [21]. Although ORP2 lacks the hallmark PIP-sensing PH domain, it encompasses an ORD domain, which in several ORPs has the capacity to accommodate and extract PI(4)P and other PIPs [6,10,34]. Noteworthy, ORP2 was previously shown to interact with the head groups of long-chain phosphoinositides *in vitro* on vesicle surfaces [24]. However, the kinetic analysis of PIP binding to the ORD of ORP2 is lacking.

To examine the specific binding affinities of PI(4)P, PI(4,5)P₂, and PI(3,4,5)P₃ to ORP2 in real-time, we used the MP-SPR NAVI 200 instrument. Recombinant pFOLD-Strep-ORP2 was attached on Switchavidin™-coated SPR sensor chips, and aqueous soluble short-chain diC8-PIPs were introduced to the protein via a continuous flow system. The binding curves for 10, 33 and 100 μM concentrations of each PIP are presented in Fig. 2. ORP2 bound PI(4,5)P₂ at 52 ± 13 μM (*n* = 3), PI(3,4,5)P₃ at 76 ± 11 μM (*n* = 3), and PI(4)P at 305 ± 155 μM (*n* = 3) apparent *K*_d (the full data is displayed in Supplemental Table S1). To investigate the PIP binding defect of ORP2(mHHK), a triple point mutant of the inositol-phosphate-binding cleft, we also purified a pFOLD-Strep fusion of this protein and subjected it to PIP binding analyses by SPR. The SPR signals of ORP2(mHHK) with all three PIPs were attenuated, but the PIP binding was not abolished (Supplemental Fig. S2), suggesting that the observed signals may involve an unspecific aspect or partial binding at a site different from the ORD pocket. Therefore, the *K*_d values determined above must be interpreted with caution.

We have previously suggested that ORP2 has the capacity to bind cholesterol [19], based on a qualitative pull-down assay in which radiolabeled cholesterol solubilized with cyclodextrin was employed as a binding substrate. The assay, however, did not allow quantitative assessment of the ligand interaction. Since the SPR assay is not compatible with the detergent conditions required to solubilize cholesterol, we employed a pull-down assay previously described by the group of N. Ridgway [33]. In this assay [³H]cholesterol is solubilized with Triton X-100 and His₆-ORP with bound [³H]cholesterol is pulled down with TALON® resin. The pFOLD-His₆-ORP2 failed to bind [³H]cholesterol over the background level of plain pFOLD-His₆ in the presence of 0.05% Triton X-100. Specific binding of cholesterol by ORP2 was, however, detected when the cholesterol was instead solubilized with 0.1% CHAPS, a zwitterionic detergent (Fig. 3A). However, saturation was not reached with the present assay, so we were unable to determine a precise *K*_d for the ORP2-cholesterol interaction.

3.2. ORP2 alone fails to enhance sterol transfer *in vitro*

To analyze whether recombinant ORP2 is capable of transferring sterol between vesicles *in vitro*, we employed a transfer assay previously employed to measure the activity of OSBP [10]. Here, the fluorescent sterol dehydroergosterol (DHE) is transferred from donor vesicles to acceptors that contain the fluorescence resonance energy transfer (FRET) partner dansyl-PE, resulting in the emission of fluorescence at 525 nm. VAPA-His₆ was added to the donor vesicles (*L*_d) containing a Ni-NTA lipid to capture the VAP, since like OSBP, ORP2 carries a FFAT motif for interaction with VAPs. PI(4)P

Fig. 2. Kinetic analysis of PI(4)P, PI(4,5)P₂, and PI(3,4,5)P₃ binding to ORP2 determined by SPR in real-time. Recombinant pFOLD-Strep-ORP2 was attached on Switchavidin™-coated SPR sensor chips, and water-soluble diC8-PIPs were directed through the flow channel at a flow rate of 20 μl/min at RT. PIP concentrations of 10 μM, 33 μM and 100 μM were used. The SPR sensorgrams were fitted with kinetic analysis of a one-site binding model with the TraceDrawer 1.7 software. The fitted binding curves of ORP2 and (A) PI(4)P, (B) PI(4,5)P₂, and (C) PI(3,4,5)P₃ are plotted against time. Mean *K*_d values ± SD (*n* = 3) are indicated.

Fig. 3. ORP2 binds cholesterol but fails to transfer it in an *in vitro* vesicle-to-vesicle assay. (A) Binding of [3 H]cholesterol by pFOLD-His₆-ORP2 and the plain pFOLD-His₆ fusion partner as a negative control; TALON[®] resin pull-down assay in the presence of 0.1% CHAPS. The x-axis depicts the concentration of cholesterol and the y-axis the bound DPM; N = 4. (B) Transfer of the fluorescent sterol DHE from donor vesicles (L_d) containing VAPA to acceptor vesicles containing 2 mol% PI(4)P (L_{a1}) by 100 nM pFOLD-His₆-ORP2, ORP2-2xStrep, and the plain pFOLD-His₆ fusion partner as a negative control. Recombinant OSBP (100 nM) was employed as a positive control. The transfer was measured as FRET signal (525 nm) between DHE and dansyl-PE in the acceptor vesicles. Also the transfer activity of OSBP in the absence of VAPA is shown. (C) Assays identical to (B) except that the acceptor vesicles contained 2 mol% PI(4,5)P₂ (L_{a2}). (D, E) Aggregation of L_d and L_{a1} (D) or L_{a2} (E) liposomes in the presence of OSBP, pFOLD-His₆-ORP2, ORP2-2xStrep or pFOLD-His₆, as measured by dynamic light scattering.

(L_{a1}) or PI(4,5)P₂ (L_{a2}) were added to the acceptor vesicles to mediate the interaction of the donor-transfer protein complex with the acceptors and to provide a potential counter-transport substrate [10]. In this assay, OSBP transferred DHE, and the reaction was enhanced by the presence of VAPA, evidencing for specific protein-mediated sterol transfer (Fig. 3B and C). However, the

pFOLD-His₆-ORP2 fusion protein failed to show DHE transfer activity (Fig. 3B and C). We suspected that the amino-terminal pFOLD-His₆ fusion partner present on the protein, which could not be removed due to protein yield and solubility problems, might inhibit a putative DHE transfer activity of ORP2. We therefore produced and purified carboxy-terminally tagged ORP2-2xStrep in

insect cells, and tested this protein in the DHE transfer assay. Similar to the pFOLD-His₆-ORP2, it failed to show transfer activity (Fig. 3B and C).

In order to investigate whether the lack of transfer activity by ORP2 might be due to inability to cluster donor (L_d) and acceptor vesicles, we carried out dynamic light scattering (DLS) experiments with vesicles incubated in the absence or presence of OSBP or ORP2. In these experiments the acceptor vesicles contained 2 mol% of either PI(4)P (L_{a1}) or PI(4,5)P₂ (L_{a2}). The results showed that OSBP induced a time-dependent clustering of the vesicles containing PI(4)P (L_{a1}) but not those containing PI(4,5)P₂ (L_{a2}) (Fig. 3D and E). However, no clustering of the liposomes was induced by the ORP2 fusion proteins. These data suggest that ORP2 alone is not able to cluster vesicles.

3.3. Role of the PIP interaction in the subcellular targeting of ORP2

To analyze the putative role of ORP2's association with cellular PIPs in the localization of the protein, the wild-type (wt) or PIP-binding attenuated (mHHK) mutant ORP2 were visualized in HeLa cells. Previous reports showed localization of ORP2 to LDs in A431, HuH7 or fatty acid-loaded HeLa TRex cells. Under certain conditions it was also detected at the PM or in the cell cortex [19,20]. In HeLa cells under standard culture conditions the wt ORP2 displayed a diffuse distribution with cytosolic, ER and occasional PM aspects, but it also frequently encircled LDs identified by internalized BODIPY 558/568 C₁₂ (Fig. 4A). Interestingly, ORP2(mHHK) displayed an enhanced targeting to LDs (Fig. 4B), and quantification confirmed a significant difference in the localization of the wt and mutant proteins (Fig. 4C). The above results are consistent with a model in which the non-LD targeting of ORP2 is mediated via phosphoinositide binding, and the ORP2(mHHK) mutant therefore shows increased secondary targeting to the LDs.

3.4. ORP2 interacts with ORP1L

We recently reported an ORP2 interactome analysis in HuH7 hepatoma cells. The analysis suggested 107 putative new interaction partners for ORP2, among which was ORP1L [21]. ORP2 and ORP1L belong to the same ORP subfamily II and share close sequence homology in their ORD domain [5]. ORP1L localizes to late endosomes (LEs) and is suggested to play multiple roles in LE motility, fusion, autophagy, and cholesterol trafficking from LEs to the ER or in the opposite direction [35–40].

The physical association of ORP2 with ORP1L was confirmed by anti-Xpress co-immunoprecipitation (IP) in HuH7 cells over-expressing Xpress-ORP2 and GFP-ORP1L constructs. The anti-Xpress-ORP2 precipitates also contained ORP1L detectable with anti-GFP antibody, while the negative controls with the plain Xpress or GFP vectors lacked the signal (Fig. 5A), supporting a specific interaction of ORP2 with ORP1L.

To further study the putative interaction of ORP2 with ORP1L, we employed the BiFC technique [41], in which ORP2 and ORP1L were each fused with a non-fluorescent partial fragment of Venus. When the two proteins interact or come to close proximity, the Venus fragments complement each other, resulting in a fluorescent signal at the subcellular compartment where the interaction occurs. Here, ORP2 and ORP1L displayed in HeLa cells a strong and specific BiFC signal at compartments which represented late endosomes based on co-localization with Rab7 (Fig. 5B). The signal did not co-localize with the LD marker BODIPY 558/568 C₁₂ (Fig. 5C). Co-localization of the ORP2-ORP1L BiFC signal with Rab7 and BODIPY 558/568 C₁₂ was quantified, showing a markedly higher Pearson's correlation coefficient for Rab7 ($\rho = 0.91$) than for the LD dye ($\rho = 0.20$) (Fig. 5B and C).

Fig. 4. ORP2 mutant with attenuated PIP binding shows enhanced targeting to lipid droplets. HeLa cells transfected for 24 h with wt ORP2 (A) or ORP2(mHHK) (B) were subjected to LD staining by using BODIPY 558/568 C₁₂. (C) Quantification of the LD localization. For each construct approximately 100 cells were analyzed visually, and the proportion of cells with prominent LD localization of ORP2 (threshold of 5 ORP2-encircled LDs) is expressed as percentage (n = 3; **p < 0.01).

To visualize the subcellular distribution of ORP2 and ORP1L in the absence of the Venus fragments employed for the BiFC, mCherry-ORP2 and GFP-ORP1L were co-expressed and imaged in live HeLa cells (Fig. 5D; Video 1; Supplemental Fig. S3). Interestingly, ORP2 and ORP1L were found to partially co-localize in perinuclear ring-like structures. These structures represent LEs/lysosomes, since they contained the internalized marker Alexa Fluor647-dextran (Fig. 5D). A majority of ORP2 and ORP1L resided on distinct organelles, which however, dynamically communicated with each other, resulting in their transient, and sometimes more stable co-localization (Videos 1A–C). Of note, ORP2 did not only localize on LEs in the presence of overexpressed ORP1L, but it was, in addition to a LD localization, frequently detectable on late endocytic compartments also when expressed alone (Fig. 5E).

Supplementary video related to this article can be found at <https://doi.org/10.1016/j.biochi.2018.12.013>.

3.5. ORP2 causes redistribution of the subcellular pools of cholesterol

The putative involvement of ORP2 in regulating cellular cholesterol homeostasis [24,25] and its interaction with ORP1L prompted us to study whether ORP2 might affect the intracellular

Fig. 5. ORP2 interacts with ORP1L. (A) Hu7 cells were transfected with pEGFP-C1-ORP1L and pcDNA4HisMax-C-ORP2 and subjected to anti-Xpress immunoprecipitation (IP). Cell lysates and the immunoprecipitates were analyzed by Western blotting with anti-ORP2 and anti-GFP antibodies. Plain pEGFP-C1 and pcDNA4HisMax-C vectors were used as negative controls indicated with (–) above the corresponding lanes. (B) BiFC: HeLa cells co-transfected with pmVN-C-ORP2 and pmVC-C-ORP1L, and stained for the late endosome marker Rab7. Pearson's correlation coefficient (ρ) for the co-localization of BiFC signal and Rab7 is displayed. (C) BiFC: Same as in (B) but lipid droplets were stained with BODIPY 558/568 C₁₂. (D) HeLa cells co-transfected with mCherry-ORP2 and GFP-ORP1L constructs, and Alexa Fluor647-dextran was internalized as a late endosome marker. (E) HeLa cells transfected with mCherry-ORP2 alone, with late endocytic compartments labeled with internalized Alexa Fluor647-dextran. Nuclei were visualized with DAPI in B–C.

distribution of cholesterol. To this end, the protein or its PIP-binding attenuated mutant ORP2(mHHK) were co-expressed in HeLa cells with a fluorescent cholesterol probe, the cholesterol-binding domain 4 of theta-toxin fused with mCherry (mCherry-D4H) [42]. In cells expressing GFP alone as a control, the D4H probe distributed between the PM and punctate/vesicular endosomes (Fig. 6A) as previously described [42]. However, in HeLa cells overexpressing wt ORP2, the D4H probe localized mainly at the PM and markedly less on the endosomal compartments as compared to

the controls (Fig. 6B). Interestingly, ORP2(mHHK) accumulated the D4H-accessible pool of cholesterol in perinuclear endosomes, drastically reducing the D4H probe intensity at the PM (Fig. 6C), suggesting a perturbation of intracellular cholesterol trafficking. Of note, the organelles with accumulated D4H probe did not co-localize with a LD marker (Fig. 6D). Quantification of D4H localization revealed significant differences between the GFP control, the wt ORP2, and ORP2(mHHK) (Fig. 6E).

To determine whether these effects of ORP2 reflect a function of

Fig. 6. Localization of the D4H cholesterol probe in HeLa cells overexpressing ORP2. The cells were transfected with the intracellular cholesterol probe mCherry-D4H and (A) pEGFP-C1 control vector (B) wild-type pEGFP-ORP2, (C) pEGFP-ORP2(mHHK), and Alexa Fluor647-dextran, or (D) pcDNA4HisMax-ORP2(mHHK) with BODIPY 493/503. The expressed ORP2(mHHK) was in (D) visualized with anti-Xpress™ antibody. (E) Quantification of the localization of D4H in endosomes (ES only), at the plasma membrane (PM only), or at both (ES & PM); approximately 30 transfected cells were calculated for each transfection ($n = 3$ for each data point); ** $p < 0.01$, *** $p < 0.001$, comparison to the GFP control.

the endogenous ORP2 protein, we carried out siRNA-mediated knock-down of ORP2 in HeLa cells. In addition, since LE accumulation of cholesterol is reported to occur in ORP1L-null cells [40], we knocked down ORP1L for a comparison. In cells transfected with non-targeting control siRNA, the D4H probe distributed between the PM and endosomes (Fig. 7A). Interestingly, when the D4H probe was imaged in the ORP2 or ORP1L knock-down cells, a majority of the probe was found accumulated in the perinuclear endosomes. Importantly, this D4H phenotype was rescued when siRNA-resistant ORP2 or ORP1L cDNA expression constructs were introduced into the respective knock-down cells (Fig. 7B), demonstrating that the perinuclear D4H accumulation was caused by the depletion of the ORPs. The D4H phenotype was quantified in both the siRNA transfected and the rescued HeLa cells, confirming significant differences in distribution of the cholesterol probe between ORP2 and ORP1L knock-down vs. control cells, as well as the rescue effects (Fig. 7C and D).

Since the D4H probe requires a threshold concentration to associate with membrane surfaces and may not faithfully reflect

the distribution of cholesterol [42], we investigated the effect of ORP2 knock-down on the subcellular distribution of cholesterol by using the cholesterol-binding dye filipin (Fig. 8A). Knock-down of Niemann-Pick C1 (NPC1) protein, a well characterized late endosomal cholesterol transporter [43], was carried out as a reference. The filipin staining revealed a similar increase of the total cellular filipin signal and the signal in LAMP1-positive late endosomes/lysosomes in both the ORP2 and the NPC1 knock-down cells (Fig. 8B and C), reinforcing the interpretation that ORP2 indeed functions in the egress of cholesterol from LEs/lysosomes.

4. Discussion

In the present study we addressed the ligand specificity and function of ORP2. Our SPR data demonstrates specific binding of PI(4,5)P₂, PI(3,4,5)P₃ and PI(4)P to ORP2. The apparent affinities are somewhat lower than those recently determined for the ORD domains of ORP5 and -8 [34]. The SPR signals of a triple point mutant affecting the inositol-phosphate-binding cleft of ORPs, ORP2

Fig. 7. ORP2 knock-down accumulates the D4H accessible cholesterol in perinuclear endosomes in HeLa cells. The cells were transfected with (A) control siRNA, ORP2 specific siRNA, or ORP1L specific siRNA. After 24-h knock-down, the cells were additionally transfected for 24 h with the mCherry-D4H cholesterol probe. Nuclei were visualized with DAPI. Western analysis of the knock-down of ORP2 and ORP1L, with β -actin probed as a loading control, is shown on the right. (B) The phenotypes were rescued by transfection (after 24-h knock-down) of siRNA resistant ORP2 and ORP1L expression constructs. (C) Quantification of the D4H phenotypes in (A). (D) Quantification of the D4H phenotypes in (B); Endosomes only (ES only), plasma membrane only (PM only), or at both (ES & PM). Approximately 30 transfected cells were calculated for each transfection ($n = 3$ for each data point); ** $p < 0.01$, *** $p < 0.001$, comparison to the CTRL siRNA (C) or to the ORP2 and ORP1L siRNA treatments (D).

H^{178–179}A, K⁴²³A (mHHK), with all three PIPs were attenuated, but the PIP binding was not abolished. This suggests that the observed signals may involve an unspecific aspect or partial binding at a site different from the ORD pocket. Therefore, the K_d values determined must be considered with caution. The pull-down assay for binding of detergent-solubilized [³H]cholesterol to ORP2 verified the specific binding earlier suggested [19], but, possibly due to the necessity of solubilizing the cholesterol with detergent, a K_d could not be determined for this interaction. It has previously been reported that the affinity of the prototype ORP, OSBP, for cholesterol is significantly lower than for oxysterol ligands such as 25OHC [44–46]. We therefore find it likely that the affinity of ORP2 for cholesterol is lower than for its high-affinity oxysterol ligand, 22(R)OHC (1.4×10^{-8} M; [19]). We have in previous studies and during

the present investigation observed that purification of fully soluble recombinant ORP2 is challenging – detailed analysis of the folding status of ORP2 produced in *E. coli* in future work is therefore warranted.

We observed that the mutant ORP2 with attenuated PIP binding, ORP2(mHHK), displayed enhanced localization to LDs, similar to the sterol-binding pocket point mutant ORP2 I249W described in Refs. [19,27]. This suggests that PIP interactions play important roles in the targeting of ORP2 to non-LD locations, and that abolishing these interactions by mutating ORP2 results in a shift of ORP2 to its second subcellular location, the LDs [19].

By using the fluorescent cholesterol-binding probe mCherry-D4H [42], we show that overexpression of wild-type ORP2 reduces the D4H cholesterol signal in endosomes, while the PIP-

Fig. 8. Late endosomal/lysosomal cholesterol accumulation upon ORP2 knock-down as confirmed by filipin staining. (A) HeLa cells transfected for 48 h with control or ORP2-specific siRNAs were stained with filipin and anti-LAMP1 antibodies. Knock-down of Niemann-Pick C1 (NPC1) was carried out as a positive control. (B) Quantification of the total cell filipin intensity (C) and that in LAMP1-positive compartments; N = 50 cells; *p < 0.05.

binding attenuated mutant ORP2(mHHK) has the opposite effect, shifting the D4H signal towards punctate/vesicular endosomal elements. This observation suggests that ORP2 has the capacity to promote the transfer of cholesterol from late endocytic compartments, consistent with its ability to enhance cellular cholesterol efflux to a variety of extracellular acceptors [24,25]. A key question was whether this is merely an overexpression artefact or whether it reflects a true function of the endogenous cellular ORP2. We therefore knocked down ORP2 to see if its depletion would cause a similar phenotype as the PIP-binding attenuated mutant. Indeed, similar to knock-down of ORP1L, which is suggested to mediate cholesterol transport between LEs and the ER [35,37,40], knock-down of ORP2 shifted the D4H and filipin towards LEs/lysosomes, suggesting that the endogenous function of ORP2 involves the egress of cholesterol from late endocytic compartments. This function appears to involve the recognition and possibly transfer of a PIP(s), since overexpression of the ORP2(mHHK) mutant resulted in the same phenotype as the knock-down. Interestingly, an earlier study implied that ORP2 plays, together with the closely related ORP1S, a role in mediating the transport of PM cholesterol to the ER and LD in A431 cells [26]. Thus, ORP2 may execute functions at two or several distinct cholesterol transport routes, considering that ORP1L is reported to play roles in both LE–ER and ER–LD transport

of cholesterol [35,37,40] and ORP5 and -8 to execute lipid transport or other functions at both ER–PM and ER–mitochondria contacts [6,47].

Concerning the reported endosomal cholesterol transport function of ORP1L, it is truly exciting that we identified ORP1L as a putative protein–protein interaction partner of ORP2 [21]. In the present study we produced more evidence for this interaction by co-immunoprecipitation of the two proteins and by BiFC, and demonstrated their partial co-localization at late endocytic compartments. This new observation is consistent with the data showing a similar shift in the distribution of the D4H cholesterol probe upon knock-down of ORP2 or ORP1L, indicating a functional relatedness or interplay between the two related proteins. In a DHE transfer assay, in which OSBP showed a significant sterol transfer activity, recombinant ORP2 preparations were devoid of activity, suggesting that ORP2 by itself may be unable to act as a sterol transporter. While OSBP induced efficient clustering of vesicles as judged from DLS experiments, ORP2 was unable to do this. These experiments should in future studies be expanded to different vesicle lipid and protein compositions. However, they indicate that, a ‘short’ ORP lacking a PH domain such as ORP2 may have a limited capacity of membrane tethering as compared to OSBP and other ‘long’ ORPs [6,10,38,48].

As a conclusion, the present study demonstrates that ORP2 binds several phosphoinositides, both PI(4)P and multiply phosphorylated species. The data on transfected cells suggests that ORP2 has an important function in the removal of cholesterol from endosomes, which depends on membrane PIPs. Moreover, ORP2 interacts with the endosomal cholesterol transporter/sensor ORP1L. The present study paves way for more detailed investigations of ORP2 function.

Authors’ contributions

A.Ko., A.A., K.T., H.K. and E.J. performed the experiments. A. Ki, P.S., J.P., T.V., V.M.O. and E.I. designed the experiments and interpreted the results together with A. Ko. V.M.O., A. Ko. and H.K. wrote the manuscript and all authors commented on the manuscript.

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Conflicts of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

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