Oxysterol-binding-protein (OSBP)-related protein 4 binds 25-hydroxycholesterol and interacts with vimentin intermediate filaments

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Oxysterol-binding protein (OSBP) is the prototypical member of a class of phospholipid and oxysterol-binding proteins that interacts with the Golgi apparatus and regulates lipid and cholesterol metabolism. As a result of recent sequencing efforts, eleven other OSBP-related proteins (ORPs) have been identified in humans. We have investigated the structure, oxysterol-binding activity, cellular localization and function of ORP4 (also designated OSBP2 or HLM), a homologue that shares the highest degree of similarity with OSBP. Two ORP4 cDNAs were identified: a full-length ORP4 containing a pleckstrin homology (PH) domain and an oxysterol-binding region (designated ORP4-L), and a splice variant in which the PH domain and part of the oxysterol-binding domain were deleted (designated ORP4-S). ORP4 mRNA and protein expression overlapped partially with OSBP and were restricted to brain, heart, muscle and kidney. Like OSBP, ORP4-L bound [³H]25-hydroxycholesterol with high affinity and specificity. In contrast, ORP4-S did not bind

[³H]25-hydroxycholesterol or [³H]7-ketocholesterol. Immunofluorescence localization in stably transfected Chinese hamster ovary cells showed that ORP4-S co-localized with vimentin and caused the intermediate filament network to bundle or aggregate. ORP4-L displayed a diffuse staining pattern that did not overlap with vimentin except when the microtubule network was disrupted with nocodazole. Oxysterols had no effect on the localization of either ORP4. Cells overexpressing ORP4-S had a 40 % reduction in the esterification of low-density-lipoprotein-derived cholesterol, demonstrating that ORP4 interaction with intermediate filaments inhibits an intracellular cholesterol-transport pathway mediated by vimentin. These studies elucidate a hitherto unknown relationship between OSBPs and the intermediate filament network that influences cholesterol transport.

Key words: cholesterol esterification, mRNA alternative splicing, tissue expression.

INTRODUCTION

With the recent availability of human genome sequences and extensive expressed-sequence-tag (EST) databases, 11 human genes have been identified that encode proteins with sequence similarity to oxysterol-binding protein (OSBP) [1–3]. While functional analysis of the gene products is incomplete, many have pleckstrin homology (PH) domains and share identity in a 300–400-amino acid region within the C-terminal oxysterol-binding domain of OSBP [4]. The founding member of the family, OSBP, was identified by Kandutsch and co-workers [5,6] as a high-affinity cytosolic receptor for a variety of oxysterol ligands, such as 25-hydroxycholesterol. It was later determined that OSBP was a cytoplasmic/vesicular protein and translocated to the Golgi apparatus in response to oxysterol binding [4]. Golgi localization of OSBP was also triggered by inhibition of cholesterol transport in the endosomal/lysosomal pathway, indicating that OSBP localization is tied intimately to cholesterol homeostasis in these organelles [7–9]. This was underscored further by studies in which overexpression of OSBP in Chinese hamster ovary (CHO) cells had pleiotropic effects on cholesterol and sphingomyelin synthesis [10,11]. In addition to these sterol-mediated effects, phorbol esters, sphingomyelin hydrolysis and elevated diglyceride levels due to phosphatidylcholine hydrolysis all enhanced OSBP interaction with the Golgi. Collectively, this shows that OSBP has a role in regulating lipid and sterol homeostasis, but its precise mechanism of action remains unknown.

The presence of multiple mammalian OSBP-like proteins hints at a shared function performed in different intracellular compartments, at different stages of development or in different tissues. Whereas some OSBP-related protein (ORP) cDNAs and genes have been identified and catalogued, only limited functional characterization has been undertaken. ORP1 and ORP2 encode peptides of 437 and 468 amino acids, respectively, that lack PH domains and are related to the yeast OSBP homologue *KES1* [2]. This extended to a functional relationship since ORP1 expression in yeast complemented *KES1* in terms of effects on cell growth and secretion. Interestingly, neither ORP1 nor ORP2 were 25-hydroxycholesterol binding proteins, and instead bound phosphatidic acid in a solid-phase assay. Another cDNA with homology to OSBP, termed HLM ('HeLa metastatic'), was cloned by differential display and shown to be up-regulated in metastatic tumour cells relative to normal human tissue [12]. This cDNA was also identified in sequence databases and designated ORP4 [1]. More recently, a full-length cDNA for ORP4/HLM was cloned and shown to encode a PH domain containing a peptide of 878 amino acids sharing extensive sequence identity with OSBP [3]. Based on oxysterol binding in crude extracts from retina, ORP4/HLM was suggested to be a 7-ketocholesterol-binding protein with negligible affinity for 25-hydroxycholesterol [3].

The yeast genome encodes seven OSBP homologues, designated *OSH1–OSH7*. A recent exhaustive analysis of the effects of disruption of individual and combinations of yeast *OSH* genes revealed a high degree of functional overlap [13]. Indeed, deletion

Abbreviations used: CHO, Chinese hamster ovary; DMEM, Dulbecco's modified Eagle's medium; EST, expressed sequence tag; LDL, low-density lipoprotein; OSBP, oxysterol-binding protein; ORP, OSBP-related protein; PH, pleckstrin homology; GST, glutathione S-transferase.

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of individual and multiple genes was without consequence, but disruption of all seven *OSH* genes was lethal. Disruption of individual and multiple *OSH* genes was accompanied by increased sterol levels and altered sensitivity to nystatin and lovastatin [13–15]. Thus each *OSH* gene performs a non-essential overlapping function, but collectively they have an essential function related to sterol or lipid metabolism. The only yeast OSBP to be characterized functionally is KES1 (OSH4), a protein that lacks a PH domain and encompasses only the C-terminal OSBP homology region. Deletion of *KES1* bypasses the requirement for *SEC14* [16], an essential gene that encodes a phosphatidylinositol/phosphatidylcholine exchange protein necessary for vesicle biogenesis at the Golgi apparatus [17]. Overexpression of *KES1* prevented rescue of cells by *SEC14* bypass mutations in the CDP-choline pathway or the PtdIns4P phosphatase, *SAC1* [16,18]. This indicates that *KES1* and *SEC14* have opposing functions related to the regulation of phosphatidylcholine and PtdIns4P metabolism and its impact on Golgi function.

A theme is emerging from studies of mammalian and yeast OSBP homologues, indicating a common function or functions related to the regulation of vesicle and lipid/sterol trafficking. However, it is unclear how these processes are affected by OSBPs or if all family members have related functions. Based on alignment of full and partial peptide sequences for 12 members of the OSBP family, OSBP and ORP4/HLM had the highest degree of sequence identity ([1], and N. D. Ridgway, unpublished work). We were thus interested in determining whether OSBP and ORP4 had related properties and functions. To this end, we undertook analysis of the gene structure, mRNA expression pattern, ligand binding and cellular localization of ORP4. We show that ORP4, like OSBP, possesses 25-hydroxycholesterol-binding activity and modulates cholesterol metabolism when overexpressed. Unlike OSBP, ORP4 splice variants were expressed in different tissues, did not translocate to the Golgi apparatus in response to oxysterols and interacted with the vimentin intermediate filament network.

EXPERIMENTAL

Materials

Vimentin monoclonal antibody V9 and nocodazole were from Sigma-Aldrich. Goat anti-mouse Texas Red and goat anti-rabbit FITC secondary antibodies were from Organon Teknika (Scarborough, ON, U.S.A.). [³H]25-Hydroxycholesterol and [³H]oleate were purchased from New England Nuclear-Mandel. [³H]7-Ketocholesterol was from American Radiolabelled Chemicals. A polyclonal antibody against ORP4 was prepared by injecting rabbits with glutathione S-transferase (GST) fused to amino acids 380–473 of ORP4-L. Human tissue Northern blots and human tissue extracts of brain and heart (Protein Medley) were purchased from Clontech. Tissue-culture media, Lipofectamine 2000 and G418 were from Gibco BRL Life Technologies. Lipoprotein-deficient fetal calf serum was prepared as described previously [19].

Cloning and construction of expression vectors

ORP4 was originally identified from the EST database (accession number AA908952). The 5' end of this EST was sequenced (A.T.C.C. accession number 427750) and used to search for related EST and genomic sequences. This led to the assembly of the full-length ORP4-L sequence (containing a PH domain and an oxysterol-binding region) based on the published human genomic sequence on chromosome 22 [20]. The ORP4-S splice variant (in which the PH domain and part of the oxysterol-

binding domain were deleted) was identified by PCR amplification of a human brain pSPORT cDNA library using an internal ORP4 oligonucleotide and a primer corresponding to the SP6 promoter in pSPORT. Several PCR products were sequenced and found to contain the exon 3 sequence fused to an additional 150–160 bp at the 5' end. There was an in-frame stop codon 7 bp 5' to the splice junction. This additional sequence was aligned with the genomic sequence on chromosome 22 (accession number AC004542) and found to encode an alternative exon (designated exon 2') between exons 2 and 3 (see Figure 1).

The ORP4-S cDNA expression vector was prepared by PCR amplification of a human brain pSPORT cDNA library using a 5' oligonucleotide adjacent to the exon 3 splice junction (see Figure 1) and a 3' oligonucleotide containing a *Hind*III site and encompassing the stop codon in exon 14. PCR products of the expected size were cloned into pcDNA3.1/V5/his-TOPO and sequenced in entirety (referred to hereafter as pcDNA-ORP4-S). Site-directed mutagenesis of methionine in ORP4-S (corresponding to positions 329 and 418 in ORP4-L) to leucine was performed using the Gene Editor system (Promega) and confirmed by sequencing.

The ORP4-L cDNA was constructed in two steps. First, the 5' end of the cDNA was amplified by PCR using a 5' oligonucleotide containing a *Hind*III site that flanked the initiation codon, and a 3' oligonucleotide primer just downstream of a unique internal *Xho*I site. PCR products were cloned into the pCR-TOPO vector and ORP4 DNAs were identified by restriction analysis and sequenced. This vector was linearized with *Xho*I and ligated with an *Xho*I fragment encompassing the 3' end of the cDNA from pcDNA-ORP4-S. This construct was then digested with *Hind*III to release the full-length ORP4L cDNA and ligated into *Hind*III-digested pcDNA3.1 (hereafter referred to as pcDNA-ORP4-L).

Cell culture and transfections

CHO cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 5% fetal calf serum (medium A). Human neuroblastoma SK-N-MC cells were cultured in DMEM with 10% fetal calf serum. Stable overexpression of ORP4 was performed by transfection of CHO cells with pcDNA-ORP4-L or pcDNA-ORP4-S by the calcium phosphate technique and selection for 2 weeks in medium A containing 500 µg of G418/ml. G418-resistant colonies were harvested by trypsin digestion and analysed for ORP4-L or ORP4-S expression by immunoblotting and immunofluorescence. Clones transfected with pcDNA-ORP4-L that were G418-resistant, but which did not express ORP4-L (as determined by immunoblotting), were used as non-expressing controls. Several CHO clones expressing ORP4 and non-expressing cells were selected for study (referred to hereafter as CHO-ORP4-S and CHO-ORP4-L). These cells were maintained in medium A containing 350 µg of G418/ml and subcultured for experiments in medium A. COS7 cells were transfected with OSBP and ORP4 cDNAs by the DEAE-dextran method [21] and harvested 48 h after transfection. Lipofectamine 2000 was used to transiently transfect CHO cells according to the manufacturer's instructions (Gibco BRL). Measurement of cholesterol esterification in CHO cells by [³H]oleate incorporation has been described previously [10].

Cellular fractionation

For analysis of OSBP and ORP expression, cells were rinsed once with 2 ml of cold PBS, and harvested by scraping in cold PBS. Cell pellets were collected by centrifugation at 3000 g for

Figure 1 Genomic and structural organization of *ORP4* splice variants

(A) The intron/exon organization of *ORP4* was determined by aligning the cDNAs for the full-length (ORP4-L) and spliced (ORP4-S) versions with the genomic sequence on chromosome 22 (accession number AC004542). (B) 5' End of the ORP4-S cDNA showing the splice junction between exons 2' and 3 (indicated by the asterisk), stop codons in frame with the initiator methionine (bold and underlined) and amino acid sequence following the initiator methionine (corresponding to Met-418 of ORP4-L). (C) The structures of ORP4-L and ORP4-S are shown compared with that of OSBP. The amino acid sequence identities between the PH and oxysterol-binding domains of OSBP and ORP4-L are 73% and 61%, respectively.

5 min and solubilized in PBS, containing 1% Triton X-100, 100 μ M PMSF and Complete protease cocktail (Boehringer Mannheim), on ice for 15 min. Detergent supernatants were collected after centrifugation at 10000 g for 10 min at 4 $^{\circ}$ C. Vimentin was extracted from Triton X-100-insoluble fractions with PBS containing 5 mM EDTA, 5 mM EGTA, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 100 μ M PMSF and Complete protease inhibitor cocktail. The detergent-soluble fraction was collected by centrifugation at 10000 g for 10 min at 4 $^{\circ}$ C.

Immunoblotting and immunofluorescence

Protein extracts (prepared as described above) were separated by SDS/PAGE, transferred to nitrocellulose membranes and incubated with primary antibodies in 20 mM Tris (pH 7.4), 150 mM NaCl, 0.1% Tween 20 and 5% (w/v) skimmed milk powder (blotting buffer) at 20 $^{\circ}$ C for 1–2 h. Nitrocellulose membranes were rinsed several times with blotting buffer and incubated with goat anti-rabbit or goat anti-mouse secondary antibodies coupled with horseradish peroxidase. Filters were developed by the chemiluminescence method according to the manufacturer's instructions (Amersham Bioscience).

Immunofluorescence of ORP4 and vimentin in overexpressing cells was similar to methods described for OSBP [4,8]. Briefly, cells cultured on glass coverslips were fixed in 3% formaldehyde and permeabilized at -20° C in 0.05% Triton X-100. All

primary and secondary antibody incubations were in PBS containing 1% BSA at 20 $^{\circ}$ C. In dual immunofluorescence-labelling studies, antibodies were added sequentially (ORP4 polyclonal antibody, goat anti-rabbit-FITC antibody, vimentin V9 monoclonal antibody and goat anti-mouse Texas Red) and slides were rinsed with PBS/1% BSA between each step. Images were taken on an Axioplan II fluorescence microscope using a 100 $\times$ objective and filter settings for FITC and Texas Red fluorescence. Images of identical fields were captured using a SPOT cooled colour digital camera and imported into Adobe Photoshop to create merged images.

Oxysterol-binding assays

COS7 cells were transfected with ORP4 or OSBP expression vectors, cultured for 48 h, harvested in Hepes (pH 7.4), 150 mM KCl, 5 mM dithiothreitol, Complete protease inhibitor cocktail and 100 mM PMSF, and homogenized by 20 passages through a 23 gauge needle. Homogenates were subjected to centrifugation at 400000 g for 15 min and supernatants (cytosol) were collected and assayed for [3 H]oxysterol binding as described previously [4,6]. Unbound [3 H]oxysterol was removed from assays by binding to charcoal/dextran followed by centrifugation. Specific binding was determined by subtracting total binding activity from binding activity in the presence of a 100-fold molar excess of unlabelled oxysterol (either 25-hydroxycholesterol or 7-ketocholesterol).

RESULTS

Structure of the *ORP4* gene and splice variants

Full-length cDNA sequences for ORP4/OSBP2 have been reported previously (accession numbers AF185696 and AF323731). Moreira et al. [3] cloned a full-length cDNA predicted to encode a protein of 878 amino acids that was designated OSBP2. In this study, we report cloning of the identical cDNA from human brain, which we have designated ORP4. The rationale for the ORP designation is to maintain consistency with the initial naming of the human OSBP family [1] and because not all ORPs bind oxysterols ([2] and this study). When the functions of more ORPs are determined, a more rational naming convention should be adopted.

The predicted amino acid sequence and intron–exon structure of ORP4 on chromosome 22 was identical to previous reports (Figure 1A). However, during the cloning of ORP4 we also identified a cDNA variant that resulted from splicing of exon 3 to an alternative exon (designated exon 2') that introduced an in-frame stop codon just upstream of the splice junction (Figures 1A and 1B). The intron between exon 2' and exon 3 is 12227 bp, whereas exon 2 and exon 3 are separated by 129059 bp. As a consequence of the upstream stop codon in ORP4-S, translation could initiate at either Met-329 or Met-418. Based on the Kozak consensus sequence at these start codons, Met-418 in exon 5 is predicted to be the preferred translational start site. Translational initiation at this methionine would produce a peptide of 460

amino acids lacking the N-terminal PH domain, as well as approx. 100 amino acids of the putative oxysterol-binding domain (Figure 1C) identified in OSBP [4].

As a first step towards characterization of ORP4, cDNAs for ORP4-L and ORP4-S were cloned into mammalian expression vectors and transiently transfected into COS7 cells. In order to detect expression of endogenous and overexpressed ORP4, we prepared a polyclonal antibody against a GST-fusion protein containing amino acids 380–473 of ORP4-L; a region that shares limited sequence identity with OSBP or other ORPs. Immunoblotting of mock-transfected COS7 cells with the ORP4 antibody revealed a single protein of approx. 108 kDa that was also evident in human neuroblastoma SK-N-MC cells (Figure 2A). Transfection of the ORP4-L cDNA into COS7 cells resulted in increased expression of a 108 kDa protein that co-migrated with endogenous ORP4-L. The lower-molecular-mass proteins at 85 and 50 kDa represent proteolysis products resulting from high-level expression of ORP4-L, as these proteins were not evident in CHO cells stably overexpressing ORP4-L (see Figure 10, below). In COS7 cells transfected with ORP4-S, a protein of 49 kDa and a cluster of proteins at 60–65 kDa were evident, but no endogenous proteins of this size were observed in COS7 or SK-N-MC cells. The molecular mass of ORP4-S from SDS/PAGE (49 kDa) was similar to the predicted molecular mass of a protein initiating from Met-418. The 60–65 kDa proteins could result from post-translational modification of ORP4-S or translational initiation from Met-329 (see below). To determine whether the proteins detected by the ORP4 antibody were specific, the same membrane as shown in Figure 2(A) was incubated with antibody pre-absorbed with the GST-ORP4 fusion protein (Figure 2B). In this case, the fusion protein effectively blocked detection of both overexpressed and endogenous ORP4. COS7 cells transfected with the OSBP cDNA expressed the 100 kDa OSBP that was detected by monoclonal 11H9 in Figure 2(C). However, the ORP4 antibody did not detect overexpressed OSBP and was specific for ORP4 species (Figure 2A).

Finally, to determine the translation-initiation site(s) in ORP4-S we expressed ORP4-S or two constructs in which Met-329 or Met-418 were converted into leucine by site-directed mutagenesis. Similar to COS7 cells, transient transfection of pcDNA-ORP4-S in CHO cells resulted in the expression of a major 49 kDa protein and minor proteins at 60 and 62 kDa (Figure 2D). ORP4-S M329L expression was similar to that of wild-type ORP4-S except for the loss of expression of the 62 kDa protein, indicating that this protein is derived from translation initiation at Met-329 (Figure 2D). Expression of ORP4-S M418L resulted in loss of expression of the major 49 kDa protein, indicating that this ORP4-S is derived from translation initiation at Met-418. The origin of the 60 kDa ORP4-S is unclear since no other methionines could account for its presence.

Figure 2 Expression of transiently transfected ORP4 in COS7 and CHO cells

COS7 cells were transfected with pcDNA-ORP4-L, pcDNA-ORP4-S, pCMV-OSBP or empty vector (mock) by the DEAE-dextran method. After 48 h, cells were harvested and expression was determined by immunoblotting with anti-ORP4 antibody (A), anti-ORP4 antibody plus GST-ORP4 peptide (B) or monoclonal 11H9 specific for OSBP (C), as described in the Experimental section. Non-transfected human neuroblastoma SK-N-MC cells were included to show the position of endogenous ORP4. Equivalent amounts of cell protein (25 µg) were loaded in each lane. (D) Methionines at positions 329 and 418 in ORP4-L were mutated to leucine in pcDNA-ORP4-L (pcDNA-ORP4-S M329L and M418L). Plasmids were transiently transfected into CHO cells, harvested after 48 h and expression of ORP4-S was detected by SDS/PAGE (25 µg of protein/lane) and immunoblotting. Nitrocellulose membranes were developed by the chemiluminescence method.

ORP4 expression in human tissues

Results shown in Figure 2 indicated that endogenous ORP4-L was expressed in COS7, SK-N-MC and CHO cells. However, endogenous ORP4-S was not detected by immunoblotting in these cells. To establish the pattern of ORP4 splice-variant expression, and to determine if the ORP4-S variant was indeed expressed, we examined ORP4 mRNA and protein in various human tissues (Figures 3 and 4). Initially, Northern blots were probed with 3' or 5' fragments of the ORP4-L cDNA that would detect both ORP4-L and ORP4-S mRNAs or only ORP4-L mRNA, respectively (Figure 3). Two mRNAs of 4.4 and 3.5 kb were detected primarily in the whole-brain and individual brain

Figure 3 Expression of ORP4 mRNA in human tissues

Northern blots of mRNA isolated from human tissues (A) or regions of the central nervous system and brain (B) were hybridized with 32 P-labelled DNA probes corresponding to nucleotides 1–376 (ORP4-5') or 761–1501 (ORP4-3') of the ORP4-L cDNA. These nucleotide probes were selected to detect only the ORP4-L mRNA (ORP4-5') or both ORP4-L and ORP4-S mRNA (ORP4-3'). OSBP mRNA was detected using a 32 P-labelled *Sma*–*Eco*RI fragment (nucleotides 258–1481) of the rabbit cDNA. Actin was detected using a cDNA probe supplied by the manufacturer (Clontech). Probes were hybridized to membranes, washed and stripped of probe between hybridizations according to the manufacturer's instructions. Blots were exposed to Kodak BioMax film at -80°C for 3–18 h.

Figure 4 Expression of ORP4-S protein in brain

Expression of endogenous ORP4 was determined by subjecting total tissue extracts (25 μg) from human heart and brain to SDS/PAGE and immunoblotting with anti-ORP4 antibody. For comparison, ORP4-S expressed in COS7 cells is shown (COS/ORP4-S). Filters were developed by the chemiluminescence method (Amersham Bioscience). The positions of ORP4-L and ORP4-S are indicated by arrowheads.

regions using the ORP4-3' probe (Figure 3). An mRNA of approx. 3.2 kb was also detected in heart using the ORP4-3' probe. A probe corresponding to 375 bp of the 5' end of

ORP4-L detected a single mRNA species of 4.4 kb, primarily in brain, and to a lesser extent in heart, skeletal muscle, spleen and kidney (Figure 3A). This shows that the 4.4 kb mRNA expressed abundantly in brain corresponds to ORP4-L, whereas the 3.5 and 3.2 kb mRNAs detected in both brain and heart, respectively, correspond to ORP4-S or another related splice variant. OSBP mRNA expression overlapped with ORP4 in heart, skeletal muscle and kidney, and to a lesser extent in liver and brain (Figure 3A). Actin expression was similar in all lanes, indicating constant mRNA loading. Brain was the only tissue where mRNA for the full-length and spliced variant of ORP4 were expressed at comparable levels (Figure 3A). The distribution of ORP4 mRNAs in regions of the brain and spinal cord is shown in Figure 3(B) using the ORP4-3' and ORP4-5' probes. The distribution of both messages was variable, with some regions of the brain expressing equal amounts of both mRNAs (cortex, and occipital, frontal and temporal lobes), primarily the 4.4 kb species (cerebellum and putamen) or little of either mRNA (medulla and spinal cord).

ORP4-S protein was not expressed in selected cultured cells (Figure 2) and mRNA was absent in most human tissues with the exception of brain and heart. To determine whether ORP4 protein was expressed in these tissues, we performed SDS/PAGE and immunoblot analysis of total brain and heart tissue with the ORP4 antibody and compared this with ORP4-S transiently expressed in COS7 cells (Figure 4). Brain, heart and COS7 cells expressed equivalent amounts of endogenous 108 kDa ORP4-L.

Figure 5 ORP4-L, but not ORP4-S, is a high-affinity 25-hydroxycholesterol-binding protein

(A) Cytosol was isolated from COS7 cells transiently transfected with expression vectors for ORP4-L, ORP4-S, OSBP or empty vector (Mock) and expression was confirmed by immunoblotting (15 µg of protein/lane) with OSBP 104 [8] or ORP4 polyclonal antibodies. (B) Cytosol (analysed for OSBP and ORP4 expression as shown in A) was assayed for specific binding of [³H]25-hydroxycholesterol (specific activity, 190 d.p.m./fmol) as described in the Experimental section. (C) Cytosol from OSBP- or ORP4-transfected COS7 cells was analysed for specific binding of 40 nM [³H]7-ketocholesterol (specific activity, 144 d.p.m./fmol) or [³H]25-hydroxycholesterol. Results in all panels are from a representative experiment that was repeated on two or three separate occasions with similar results.

In addition to ORP4-L, a 49 kDa protein similar to the transfected ORP4-S was detected in brain. Heart expressed a protein of higher molecular mass (approx. 55 kDa) and a cluster of proteins at 60–65 kDa. The 55 kDa protein could represent another ORP4-S variant since this tissue expressed an ORP4-S mRNA smaller than that observed in brain (Figure 3A). The 60–65 kDa proteins were abundant in heart and reacted non-specifically with the secondary antibody or preimmune serum. This confirms that ORP4-S mRNA and protein are predominately expressed in brain and heart.

Oxysterol-binding activity of ORP4

The high degree of sequence similarity between OSBP and ORP4 [1,3] suggested that ORP4 bound oxysterols. Indeed, it was

Figure 6 Competitive binding of oxysterols is similar for OSBP and ORP4-L

Cytosols prepared from COS7 cells transiently transfected with OSBP (A) or ORP4-L (B) were incubated with 10 nM [³H]25-hydroxycholesterol (specific activity, 190 d.p.m./fmol) and increasing concentrations of unlabelled oxysterols for 12 h at 4 °C. Specific binding to OSBP was determined after removal of unbound [³H]25-hydroxycholesterol by charcoal–dextran as described in the Experimental section. Results are the means from duplicate determinations in a representative experiment repeated three times with similar results. Abbreviations: 25-OH, 25-hydroxycholesterol; 7-Keto, 7-ketocholesterol; 20-OH, 20-hydroxycholesterol; 22(R)-OH, 22(R)-hydroxycholesterol; 22(S)-OH, 22(S)-hydroxycholesterol; 7-OH, 7-hydroxycholesterol.

reported previously that OSBP2/ORP4 was a 7-ketocholesterol-binding protein [3]. However, this result was based on binding activity in crude extracts of monkey retina. It is our experience that oxysterol-binding activity can be detected, studied and attributed to a specific protein more accurately by overexpression of the cDNA in cultured cells [4,10,22]. Thus we transiently overexpressed ORP4-L, ORP4-S and OSBP in COS7 cells and compared [³H]25-hydroxycholesterol and [³H]7-ketocholesterol activities in cytosolic extracts (Figure 5). Abundant and comparable amounts of OSBP, ORP4-L and ORP4-S were expressed in the cytosol from transfected COS7 cells, as indicated by immunoblotting using a polyclonal antibody raised against the PH domain of OSBP [8], which cross-reacted with ORP4-L (OSBP 104 antibody), and the ORP4-specific antibody (Figure 5A). When these extracts were assayed for binding activity using increasing concentrations of [³H]25-hydroxycholesterol, it was apparent that ORP4-L displayed high-affinity binding similar to OSBP (Figure 5B). In contrast, [³H]25-hydroxycholesterol binding to cytosol from ORP4-S-transfected cells was absent (similar to mock-transfected controls). The dissociation constant for 25-hydroxycholesterol binding to ORP4-L was 10–15 nM, similar

Figure 7 Intracellular localization of ORP4 was not affected by 25-hydroxycholesterol

CHO cells were transiently transfected with pcDNA-ORP4-L or pcDNA-ORP4-S using Lipofectamine 2000. After transfection (48 h), cells were treated with 2.5 μ g of 25-hydroxycholesterol/ml in medium A for 2 h (25-OH Chol), fixed and processed for immunofluorescence using the ORP4 antibody and FITC-labelled goat anti-rabbit secondary antibody as described in the Experimental section.

to that reported for OSBP [4,10]. When [3 H]ketocholesterol and [3 H]25-hydroxycholesterol (40 nM) binding was compared (Figure 5C), it was apparent that [3 H]7-ketocholesterol-binding activity in cytosols from ORP4-L-, ORP4-S- and OSBP-transfected COS7 cells was similar to mock-transfected cell extracts (in two similar experiments, [3 H]7-ketocholesterol binding to OSBP and ORP4 was less than mock values). In contrast, [3 H]25-hydroxycholesterol binding to ORP4-L and OSBP was 8–15-fold above background (mock transfectants), respectively.

The binding specificities of OSBP and ORP4-L were compared in more detail with a competition assay using a variety of unlabelled oxysterols (Figure 6). In these experiments, COS7 cytosol extracts from cells transfected with OSBP and ORP4-L expression vectors were incubated with 10 nM [3 H]25-hydroxycholesterol and increasing concentrations of unlabelled oxysterols. The extent of displacement of [3 H]25-hydroxycholesterol by unlabelled oxysterols provides an indirect measure of binding when radiolabelled ligands are not available [22]. However, without complete information on OSBP- and ORP4-L-binding sites for these ligands, competition could occur by indirect means. As expected, almost 100 % of [3 H]25-hydroxycholesterol was displaced from OSBP (Figure 6A) and ORP4-L (Figure 6B) by 100 nM unlabelled 25-hydroxycholesterol. The displacement curves were similar for OSBP and ORP4-L. 20-Hydroxycholesterol at a concentration of 100 nM was 70 % effective in displacing 25-hydroxycholesterol, while 7-ketocholesterol was less effective in displacing labelled oxysterol from ORP4-L.

Other oxysterols were only effective in displacing 25-hydroxycholesterol when present at high concentrations (0.5–2 μ M). Collectively, the results in Figures 5 and 6 show that ORP4-L and OSBP are high-affinity 25-hydroxycholesterol-binding proteins and have similar oxysterol-displacement curves, whereas ORP4-S is inactive for oxysterol binding.

Co-localization of ORP4 with the vimentin intermediate filament network

Intracellular localization was determined by indirect immunofluorescence in CHO cells transiently transfected with expression vectors for ORP4-L, ORP4-S or empty vector (mock transfected) and treated with 25-hydroxycholesterol in the medium for 2 h (Figure 7). There was virtually no immunofluorescence associated with mock-transfected cells incubated with the ORP4 antibody, indicating that expression of endogenous ORP4 was below the limits of detection. In contrast to OSBP, which is in a Golgi/vesicular compartment [4], overexpressed ORP4-L was widely dispersed throughout the cell and was not concentrated in any particular structure(s). In contrast, overexpressed ORP4-S was localized to unusual structures close to the nucleus, and in thin filaments or fibres that projected from these structures around the nucleus and to the periphery of the cell. The addition of 25-hydroxycholesterol to the culture medium for 2 h did not alter localization of either ORP4-L or ORP4-S. Other oxysterols

Figure 8 ORP4-S localizes with vimentin intermediate filaments

CHO-ORP4-L (ORP4-L), CHO-ORP4-S (ORP4-S) or non-expressing CHO cells (Mock) were cultured in medium A on glass coverslips, and processed for indirect immunofluorescence of ORP4 (FITC) and vimentin (Texas Red) as described in the Experimental section.

Figure 9 Localization of ORP4 with vimentin in nocodazole-treated cells

Experiments were performed as described in the legend to Figure 8, with the exception that cells were treated with 2 μ g of nocodazole/ml for 1 h prior to processing for immunofluorescence.

Figure 10 ORP4 and vimentin are associated with the Triton X-100-resistant fraction of CHO cells

Non-expressing controls (CHO), CHO-ORP4-L and CHO-ORP4-S cells were extracted with buffer containing 1% Triton X-100, and detergent-soluble and -insoluble fractions were isolated as described in the Experimental section. Triton-soluble (A) and -insoluble (B) proteins were separated by SDS/PAGE and immunoblotted for ORP4 or vimentin (V9 monoclonal antibody). Equivalent amounts of protein (5 μ g) were loaded in each lane.

(listed in the legend to Figure 6) also had no effect on ORP4 localization. In parallel experiments using OSBP overexpressing cells, we confirmed that 25-hydroxycholesterol promoted translocation of OSBP to the Golgi apparatus under the same conditions described in Figure 7 (results not shown).

The filamentous staining pattern for ORP4-S indicated an interaction with the cytoskeletal network, and with intermediate filaments in particular [23]. Vimentin is highly expressed in cultured cells and mammalian tissues [23], but is not essential for viability in cultured cell models [24,25] and vimentin-null mice [26]. Whereas vimentin serves as a scaffold for numerous proteins involved in metabolism, vesicle trafficking and signalling [27–31] and confers structural integrity to cells [32], its function has not been established firmly. Since the localization of ORP4-S resembled disorganized vimentin [27,33], we examined co-localization by dual immunofluorescence of ORP4-L or ORP4-S with vimentin (Figure 8). These experiments used CHO cells stably transfected with ORP4-L (CHO-ORP4-L) or ORP4-S (CHO-ORP4-S), or non-expressing controls. In control CHO cells, vimentin (Texas Red immunofluorescence) was dispersed throughout the cells in an irregular network. In ORP4-S-overexpressing cells, vimentin filaments were more disorganized and showed evidence of ‘bundling’ or aggregation, particularly around the nucleus, when compared with non-expressing cells. ORP4-S (FITC immunofluorescence) was concentrated in filamentous bundles with vimentin, but displayed limited co-localization in areas where vimentin was not aggregated. In CHO-ORP4-L cells, vimentin was localized to a filamentous network (similar to mock cells) that extended throughout the cell. There was no appreciable bundling of filaments, and little or no overlap with ORP4-L.

The apparent collapse and bundling of vimentin filaments shown in ORP4-S-expressing cells (Figure 8) is reminiscent of vimentin organization in which the microtubule network is

depolymerized by nocodazole [33], suggesting that ORP4 and microtubules might also be associated. To determine whether an intact microtubule network is required for ORP4 to interact with the vimentin network, ORP4-transfected cells were treated with nocodazole, and ORP4 and vimentin were co-localized by indirect immunofluorescence (Figure 9). The effectiveness of nocodazole was confirmed by the virtual absence of a microtubule network in cells treated with the drug for 1 h (results not shown). Compared with untreated control cells (see Figure 8), vimentin displayed capping or bundling around the nucleus in response to nocodazole treatment. When CHO-ORP4-S cells were treated with nocodazole, vimentin was also localized to prominent bundles around the nucleus that co-localized with ORP4-S. However, the localization of ORP4-S with nocodazole treatment was similar to untreated CHO-ORP4-S cells (see Figure 8), indicating that ORP4-S co-localization with vimentin is not dependent on the microtubule network, and alone caused a nocodazole-like phenotype. ORP4-L localization in nocodazole-treated cells was similar to untreated controls (see Figure 8), except that ORP4-L was localized to zones adjacent to the plasma membrane in nocodazole-treated cells. Unlike the results in ORP4-S cells, nocodazole did not induce extensive bundling of vimentin in ORP4-L-overexpressing cells. Instead, vimentin was localized diffusely around the nucleus and in zones adjacent to the plasma membrane that overlapped with ORP4-L.

The vimentin network is a stable polymeric structure that resists extraction with 1% Triton X-100. As shown in Figure 10, ORP4-L and ORP4-S were recovered in a Triton X-100-resistant fraction of stably transfected CHO cells that was highly enriched in vimentin. Based on total protein recovery, approx. 20% of ORP4-L and ORP4-S were recovered in the vimentin-enriched fraction, with the remainder extracted by 1% Triton X-100. Compared with non-expressing control cells, ORP4-L and ORP4-S overexpression did not alter expression or Triton solubility of vimentin. It is also notable that stable expression of ORP4-L in CHO cells was not accompanied by the extensive proteolysis observed in COS7 cells, and ORP4-S expression was restricted primarily to the 49 kDa form.

ORP4-S expression decreased low-density lipoprotein (LDL)-derived cholesterol esterification

Previous studies have shown that vimentin intermediate filaments are associated with and influence the metabolism and trafficking of cellular sphingolipids and sterols [25,34,35]. ORP4 could act as an auxiliary factor that interacts with vimentin and regulates transport of these lipid and sterols. To test this, we examined LDL-derived cholesterol esterification in CHO cells over-expressing ORP4. The overexpressing cell lines and controls described in Figures 8 and 9, as well as one additional cell line for each, were cultured in lipoprotein-free medium with or without LDL, and cholesterol esterification (Figure 11A) and triglyceride synthesis (Figure 11B) were measured by [3 H]oleate labelling. The basal cholesterol-esterification rate in cells cultured in delipidated medium for 18 h was similar in control and over-expressing cell lines. Treatment with human LDL for 6 h resulted in increased [3 H]oleate incorporation into cholesterol ester in all cell lines. However, compared with controls, two cell lines expressing ORP4-S had esterification rates that were reduced significantly, by 40%. The ORP4-L-expressing lines displayed a 15–20% reduction in esterification rates that was not significant. [3 H]Oleate incorporation into triacylglycerol (Figure 11B) and total phospholipids (results not shown) was variable between the cell lines and was not affected significantly by ORP4 expression and LDL.

Figure 11 Overexpression of ORP4-S inhibits esterification of LDL-derived cholesterol

Two independently isolated CHO cell lines stably expressing ORP4-S, ORP4-L or non-expressing controls were cultured in DMEM containing 5% lipoprotein-deficient serum for 18 h. Experiments were initiated by treatment with (+ LDL, hatched bars) or without (NA, solid and stippled bars) 50 μ g of LDL/ml for 6 h. For the final hour of LDL treatment, cellular lipids were labelled with 100 μ M [3 H]oleate (3.18 d.p.m./pmol). Cells were harvested and [3 H]oleate incorporation into cholesterol ester (A) and triacylglycerol (B) was determined as described in the Experimental section. Results are the means $\pm$ S.D. from 3–6 separate experiments. The levels of expression of ORP4-S and ORP4-L were similar in the two cell lines tested, as determined by immunoblotting and immunofluorescence. * P < 0.01 versus CHO-control cells + LDL. Abbreviations: CE, cholesterol ester; TAG, triacylglyceride.

DISCUSSION

The availability of the human genome sequence and extensive EST databases has lead to the identification of new members of the *OSBP* gene family, which now comprises at least 12 members in humans ([1–3], and N. D. Ridgway, unpublished work). Here we report the characterization of one member of this family that shares a high degree of similarity with the founding member of this family, OSBP [3]. Despite sharing similarities in sequence and oxysterol-binding activity with OSBP, the *ORP4* gene encoded two splice variants that were localized in different compartments of the cell and were expressed in different human tissues. Like OSBP [10], ORP4-S expression decreased cholesterol esterification in response to LDL. This was not mediated via interaction with the Golgi apparatus, but appeared to involve the vimentin intermediate-filament network, which is known to regulate cholesterol transport from the lysosomal/endosomal pathway [25,36].

Contrary to a recent report [3], we show that full-length ORP4-L, like OSBP, is a high-affinity 25-hydroxycholesterol-binding protein. Direct binding assays using [3 H]25-hydroxycholesterol and competition assays using unlabelled oxysterols,

such as 20-hydroxycholesterol, 7-ketocholesterol and 22(S)- and 22(R)-hydroxycholesterol, indicated ORP4-L expressed in COS7 cells bound 25-hydroxycholesterol with high affinity and specificity. 7-Ketocholesterol was effective in competing for 25-hydroxycholesterol binding when added in 10-fold excess. However, direct binding of 40 nM [3 H]7-ketocholesterol by ORP4-L and OSBP was trivial relative to 25-hydroxycholesterol. As mentioned, this is in direct contrast to an activity detected in retina that co-migrated with ORP4 and bound 0.1 nM [3 H]7-ketocholesterol but not 25-hydroxycholesterol [3]. In that study, binding specificity was based on co-elution of labelled oxysterol and ORP4 (assessed by immunoblotting) in monkey retina fractions incubated with labelled oxysterols and fractionated by HPLC. The potential disadvantages of this approach are the presence of contaminating protein(s) that bind oxysterols, insufficient oxysterol-binding protein in the extract for detection, or substances in the extracts that interfere with oxysterol binding. These problems were overcome by overexpressing ORP cDNAs in cultured cells and comparing binding activity to mock- and OSBP-transfected cells. In this manner we could directly relate binding activity to the presence of ORP4 proteins.

In addition to the full-length protein, the *ORP4* gene encoded a truncated splice variant missing the N-terminal PH domain and part of the oxysterol-binding domain. Based on deletion and truncation mutants of OSBP, the 25-hydroxycholesterol-binding region was assigned to a broad region in the C-terminal 500 amino acids [4]. ORP4-S, which initiates primarily at Met-418 of ORP4-L when expressed in COS7 and CHO cells, would have part of this region deleted, resulting in a loss of oxysterol binding. It is also possible that the lack of oxysterol binding by ORP4-S was due to a post-translational modification or the presence of an inhibitor in cell extracts. When expressed in COS7 cells, ORP4-S displayed aberrant-molecular-mass species that could not be entirely accounted for by an alternate translational start site at Met-329 (Figure 2). However, these high-molecular-mass species were reduced in stably expressing CHO cells (Figure 10) and absent on immunoblots of ORP4-S expressed in brain and heart (Figure 4), indicating that they were probably an artifact of high-level expression in cultured cells.

The absence of PH and oxysterol-binding domains in ORP4-S had another functional consequence; localization and modification of the vimentin intermediate-filament network. Since ORP4-L did not co-localize with vimentin under normal conditions, it would appear that the PH and/or oxysterol-binding domains are negative regulators of this interaction. This is strikingly similar to OSBP interaction with the Golgi apparatus. In that case, the oxysterol-binding domain prevented OSBP translocation to the Golgi apparatus unless deleted or bound to oxysterol [4]. In a similar manner, the absence of a functional oxysterol-binding domain on ORP4-S, as shown by a lack of 25-hydroxycholesterol binding, could enhance interaction with vimentin. However, unlike OSBP interaction with the Golgi apparatus [10,37], ORP4-S interaction with vimentin did not require a PH domain, which was missing in the splice variant. A lack of requirement for the PH domain was not surprising since the PH domain from OSBP and other proteins are primarily involved in protein–phosphoinositide interactions [37,38]. The fact that ORP4-L was not induced to localize with vimentin in the presence of 25-hydroxycholesterol or other oxysterols suggests that the PH domain prevents interaction with vimentin, possibly by keeping ORP4-L tethered in another compartment.

Unlike OSBP, ORP4-L does not translocate between cellular compartments in response to oxysterols or other conditions that alter cholesterol homeostasis or transport (i.e. sterol depletion, inhibition of LDL-derived cholesterol transport and sphingo-

myelinase treatment; results not shown). However, ORP4-L was observed to partially localize with vimentin when the microtubule network was disrupted with nocodazole. Due to the intimate association of the vimentin and microtubule networks, disruption of the latter resulted in collapse of the vimentin network to aggregated structures around the nucleus ([33] and Figure 8). In cells expressing ORP4-L, this response was prevented and vimentin and ORP4-L were present in a diffuse ring around the nucleus and in patches at the cell periphery. In contrast, ORP4-S promoted vimentin bundling and was not affected by nocodazole. This suggests that ORP4-L, unlike ORP4-S, interacts with vimentin in a manner that is dependent on the microtubule network.

Although expression of vimentin and other intermediate-filament networks is widespread, function remains obscure. Studies in cultured cells devoid of vimentin noted decreased glycosphingolipid and sterol synthesis that was attributed to a role in lipid transport [25,34,35]. Thus vimentin could serve as a scaffold or track to support transport of specific vesicle populations or maintain organellar structure [27,29]. This is a highly redundant or non-essential process that is shared by a closely associated microtubule network and other intermediate-filament networks. Indeed, the absence of vimentin does not affect Golgi ultrastructure, secretion or endocytosis [26,27,29], suggesting that a limited subset of transport events are involved. Our finding that ORP4-S-expressing cells had a 40 % decrease in esterification of LDL-derived cholesterol suggests that ORP4-S interacts with the vimentin network to modify cholesterol transport. Hydrolysis of LDL-derived cholesterol ester occurs in the late endosomes and cholesterol is subsequently transported to the endoplasmic reticulum where it is re-esterified by acylCoA:cholesterol acyltransferase (reviewed in [39]). ORP4-S overexpression and decreased vimentin expression [25] had a similar effect on cholesterol esterification, suggesting that ORP4-S effectively inhibits the activity of endogenous vimentin by promoting the aggregation or bundling of the intermediate-filament network (see Figures 7 and 8). ORP4-L expression had a minor effect on cholesterol esterification, consistent with a limited interaction with vimentin under normal conditions. Interestingly, OSBP expression in CHO cells also inhibited cholesterol esterification and acylCoA:cholesterol acyltransferase activity [10]. Whether this involves direct or indirect interactions of OSBP with vimentin or ORP4 is unknown.

Although few OSBP-related proteins have been characterized, the identification of ORP4 as a 25-hydroxycholesterol-binding protein implicated in sterol regulation or transport suggests a common function for some family members. However, it appears that ORP4 and OSBP have discrete functions in this pathway based on differences in cellular localization and tissue distribution. Continued characterization of other OSBP homologues will provide further clues to the function of this diverse class of proteins.

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