OSBP Negatively Regulates ABCA1 Protein Stability*5

Kristin Bowden1 and Neale D. Ridgway2

From the Departments of Pediatrics and Biochemistry and Molecular Biology, Atlantic Research Centre, Dalhousie University, Halifax, Nova Scotia B3H 4H7, Canada

Oxysterol binding to liver X receptors (LXR) increases the transcription of genes involved in cholesterol efflux and disposal, such as ABCA1 (ATP-binding cassette transporter A1). Other cytoplasmic sterol-binding proteins could interact with this pathway by sequestering or delivering substrates and ligands. One potential regulator is OSBP (oxysterol-binding protein), which is implicated in the integration of sterol sensing/transport with sphingomyelin synthesis and cell signaling. Since these activities could impact the cholesterol efflux pathway, we examined whether OSBP was involved in LXR regulation and in expression and activity of ABCA1. Suppression of OSBP in Chinese hamster ovary cells by RNA interference resulted in increased ABCA1 protein expression and cholesterol efflux activity following induction with oxysterols or the synthetic LXR agonist TO901317. OSBP knockdown in J774 macrophages also increased ABCA1 expression in the presence and absence of LXR agonists. OSBP depletion did not affect ABCA1 mRNA levels or LXR activity. Rather, OSBP silencing increased the half-life of ABCA1 protein by 3-fold. Sphingomyelin synthesis was suppressed in OSBP-depleted cells treated with 25-hydroxycholesterol but not TO901317 or 22-hydroxycholesterol and did not correlate with ABCA1 stabilization. Moreover, co-transfection experiments revealed that reduction of ABCA1 protein by OSBP was prevented by a mutation in the sterol-binding domain but not by mutations that abrogated interaction with the Golgi apparatus or endoplasmic reticulum. Thus, OSBP opposes the activity of LXR by negatively regulating ABCA1 activity in the cytoplasm by sterol-binding domain-dependent protein destabilization.

Disposal of excess cholesterol by peripheral tissues and the liver is mediated by liver X receptors (LXR),5 ligand-activated nuclear transcription factors that increase expression of genes encoding ATP-binding cassette proteins (1–4), bile acid metabolic enzymes (reviewed in Ref. 5), and apolipoproteins (6). LXR is activated by oxysterol derivatives of cholesterol in vivo and is thus positioned to sense the cellular levels of cholesterol and regulate genes required for its elimination (7). The ABCA1-dependent efflux of cholesterol and phospholipids to extracellular apolipoprotein A1 (apoAI) to form nascent high density lipoprotein is the initial and limiting step in the reverse cholesterol transport pathway (reviewed in Ref. 8). Although transcriptional induction of ABCA1 by LXR is a key event in this process, it is now recognized that regulation of ABCA1 protein stability in the plasma membrane (PM) and endosomes is also involved. ApoAI binds and stabilizes ABCA1, resulting in increased PM localization and decreased degradation by thiol proteases (9, 10). Phosphorylation of a PEST sequence in the large cytoplasmic loop of ABCA1 mediates internalization and subsequent degradation by calpain (11–13). Phosphorylation of the PEST sequence is inhibited by apoAI, resulting in increased surface localization and stabilization. ABCA1 also interacts with β1- and α1-syntrophin, scaffolding proteins that regulate transport through linkage to the cytoskeleton, resulting in stabilization at intracellular sites and the PM (14, 15). Thus, distribution of ABCA1 between the PM and endosomes affects protein stability and efficiency of cholesterol efflux to apoAI.

Other sterol-binding proteins could affect cholesterol homeostasis by sequestration or delivery of oxysterol ligands to LXR or by influencing the supply of cholesterol or phospholipids to ABCA1 for efflux to apoAI. A class of binding proteins that could fulfill these functions is the OSBP (oxysterol-binding protein) and ORP (OSBP-related) gene family, which are expressed across eukaryotic phyla and consist of 12 mammalian and seven yeast members (16, 17). OSBP and ORPs are characterized by a C-terminal OSBP homology domain that binds oxysterols and cholesterol (18–20). The majority of family members also contain pleckstrin homology (PH) and FFAT (two phenylalanines in an acidic tract) domains that mediate interaction with phosphatidylinositol phosphates (21, 22) and the resident ER protein vesicle-associated membrane protein-associated protein (VAP) (23, 24), respectively. The PH and FFAT domain of OSBP facilitate partitioning between the ER and Golgi apparatus in response to oxysterol and cholesterol binding (23, 25, 26). Sterol-dependent partitioning of OSBP between the Golgi and ER does not appear to be directly involved in cholesterol regulation, since depletion by RNA interference did not affect the

short hairpin RNA; shNT, nontargeting short hairpin RNA; siOSBP, OSBP-specific siRNA; siNT, siRNA against nontargeting control.

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1 Recipient of a Nova Scotia Health Research Foundation studentship.

2 To whom correspondence should be addressed: Atlantic Research Centre, 5849 University Ave., Dalhousie University, Halifax, Nova Scotia B3H 4H7, Canada. Fax: 902-494-1394; E-mail: nridgway@dal.ca.

3 The abbreviations used are: LXR, liver X receptor(s); apoAI, apolipoprotein AI; DMEM, Dulbecco’s modified Eagle’s medium; FFAT, two phenylalanines in an acidic tract; 25OH, 25-hydroxycholesterol; 22OH, 22(R)-hydroxycholesterol; MCD, methyl-β-cyclodextrin; PH, pleckstrin homology; PM, plasma membrane; RA, 9-cis-retinoic acid; siRNA, short interfering RNA; VAP, vesicle-associated membrane protein-associated protein; ER, endoplasmic reticulum; CHO, Chinese hamster ovary; shOSBP, OSBP-specific siRNA.

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The on-line version of this article (available at http://www.jbc.org) contains supplemental Fig. 1.

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sterol-regulatory element-binding protein pathway and cholesterol ester synthesis (27, 28). Rather, OSBP mediates the oxysterol-dependent regulation of the ceramide transfer protein (CERT) and sphingomyelin synthesis in the Golgi apparatus (28), indicating a sensing function for integration of cellular cholesterol and sphingomyelin levels.

The pleiotropic effects of yeast OSH deletions (29) and mammalian ORP overexpression (22, 30, 31) on cholesterol homeostasis also suggest a functional interaction at the level of cholesterol transport and/or regulation of transport machinery. Indeed, yeast Osh4p binds and transports sterols in vitro and has been implicated along with other OSH proteins in sterol transport in vivo (32). Recent evidence suggests that the observed effects of ORPs on cholesterol homeostasis in mammalian cells could be mediated directly or indirectly through LXR. Overexpression of ORP11 in COS cells increased LXRα activity, based on a reporter gene assay (33). However, transgenic expression of ORP11 in macrophages implanted in the bone marrow of LDL−/− mice resulted in decreased expression of the LXR target genes ABCG1, apoE, and ABCG5 (34). Adenoviral expression of OSBP in mouse hepatocytes did not affect mRNA for ABCA1 and ABCG1 but up-regulated SREBP1c (sterol-regulatory element-binding protein 1c) expression and processing (35). Recently, ORP8 was shown to negatively regulate ABCA1 transcription by a mechanism involving DR4 and E-box elements (36). Since ORP8 is anchored to the ER by a C-terminal transmembrane domain, transcriptional inhibition is probably indirect and related to the sterol binding or metabolic activity of this ORP.

The cholesterol- and oxysterol-binding activity of OSBP and resultant activation of sphingomyelin synthesis in the Golgi apparatus have the potential to impact on ABCA1 expression and activity. To test this, RNA interference was used to silence OSBP, and resultant effects on ABCA1 expression and activity were determined. Results show that OSBP negatively regulates ABCA1 expression and cholesterol efflux activity by decreasing protein stability. This effect was not correlated with sphingomyelin synthesis or interaction of OSBP with the ER or Golgi apparatus but was dependent on OSBP sterol-binding activity. We conclude that OSBP opposes the activity of LXR by negatively regulating ABCA1 protein stability in the cytoplasmic compartment.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfection**—CHO cells were cultured in Dulbecco’s modified Eagles medium (DMEM) containing 10% fetal calf serum and proline (33 μg/ml) (medium A) at 37 °C in 5% CO2. J774.1 macrophages were cultured in DMEM with 10% fetal calf serum. CHO cells stably expressing a vector encoding a short hairpin RNA targeting OSBP or a nontargeting control were cultured in medium A with G418 (350 μg/ml) (28). For cholesterol efflux experiments, CHO cells were cultured in DMEM, 5% (v/v) lipoprotein-deficient serum, and proline (medium B). Cells received concentrated stock solutions of 22(R)-hydroxycholesterol (22OH) and 25-hydroxycholesterol (25OH), TO901317 or 9-cis retinoic acid (RA) such that the final concentration of DMSO or ethanol solvent in media did not exceed 0.1% (v/v).

CHO cells were transfected with murine pABCA1-FLAG (Dr. Nan Wang, Department of Medicine, Columbia University, NY) and pOSBP using Lipofectamine 2000 (Invitrogen). CHO and J774 cells were transiently transfected with a short interfering RNA (siRNA) against OSBP (siOSBP2) or nontargeting controls (siNT) purchased from Dharmacon Inc. (Lafayette, CO) using Trans-IT TKO transfection reagent (Mirus Corp., Nepean, Canada), as previously described (28).

**Immunoblotting**—Cells were washed twice with PBS, scraped from the dish, sedimented at 10,000 × g for 1 min, and resuspended in 100 μl of Triton-X buffer (20 mM HEPES (pH 7.4), 5 mM KCl, 5 mM MgCl2, 0.5% Triton X-100) on ice for 10 min. Supernatants were collected after centrifugation for 10 min at 10,000 × g and analyzed by SDS-PAGE and immunoblotting. Analysis of the supernatant and pellet fractions by immunoblotting indicated that >95% of the ABCA1 protein was found in the Triton-soluble (supernatant) fraction. To preserve the integrity of ABCA1, samples were not heated prior to resolution of equivalent amounts of protein (15 μg) by SDS-PAGE on discontinuous 6–10% gels. Following transfer to nitrocellulose, filters were probed with primary antibodies against ABCA1 (Novus Biologicals, Littleton, CO), OSBP, and actin, followed by goat anti-rabbit or goat anti-mouse horseradish peroxidase-conjugated secondary antibodies. Proteins were visualized by chemiluminescence and exposed to film, and expression was quantified by densitometry using ImageJ 1.38 (National Institutes of Health, Bethesda, MD).

**Cell Surface Biotinylation**—Proteins on the surface of CHO cells were biotinylated, harvested, and purified on streptavidin resin using a cell surface isolation pack (Pierce) and original methods (37). Briefly, after surface biotinylation and quenching, cells were lysed, and debris was removed by centrifugation at 10,000 × g for 10 min. Biotinylated proteins in the supernatant (500 μl) were bound to a streptavidin column and eluted in 250 μl of SDS sample buffer. The total and cell surface fraction were treated with 50 mM dithiothreitol to remove biotin, resolved by SDS-PAGE, and immunoblotted using antibodies against ABCA1, OSBP, actin, protein kinase D, and AKT.

**Cholesterol Efflux Assays**—Twenty-four hours after siRNA transfection, CHO cells received medium B, containing cholesterol (10 μg/ml) and [3H]cholesterol (0.3 μCi/ml) together with 22OH (1 μg/ml) and 10 μM RA, 1.0 μM TO901317, and 10 μM RA or solvent controls. After 18 h, cells were incubated for 1 h with medium B and then washed twice with warm DMEM. Cells were then incubated in DMEM or DMEM with 20 μg of apoAl/ml (Calbiochem/EMD Biosciences Inc. Mississauga, Canada) for 1 h at 37 °C. Radioactivity in media (subjected to centrifugation at 10,000 rpm for 1 min to remove cellular debris) and cells (lysed in 0.5 N sodium hydroxide for 30 min) were quantified by liquid scintillation counting. The percentage of cholesterol efflux from cells was calculated based on radioactivity in the media divided by the total (cells and media). APOAI-specific efflux was determined by subtracting cholesterol efflux in DMEM.

Extraction of cholesterol from CHO-K1 cells by methyl-β-cyclodextrin (MCD) followed a similar protocol as described above except that after [3H]cholesterol loading, cells were treated with 2 mM MCD in DMEM. Cell lysate and medium fractions were collected, and [3H]cholesterol was quantified as described above.
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**LXR Reporter Gene Expression**—CHO cells stably expressing a nontargeting short hairpin RNA (shNT) and an OSBP-specific short hairpin RNA (shOSBP) (seeded in 12-well plates) were co-transfected with pGL3-LXREX3 (provided by Dr. Chris Sinal, Dalhousie University) (5 ng/well) and pCMV-βgal (20 ng/well). After 24 h, cells were treated with solvent alone, 22OH (1.0 μg/ml) plus 10 nM RA for 18 h. Cell lysates were resolved by 6–10% SDS-PAGE and analyzed by immunoblotting using primary antibodies against OSBP, ABCA1, and tubulin (Tub). B, shNT and shOSBP cells were treated with solvent (NA) or 22OH (1.0 μg/ml) with and without RA for 0–12 h. Cell lysates were analyzed by immunoblotting with primary antibodies against ABCA1 and actin. The extent of OSBP knockdown in shOSBP cells was similar to results in A. C, CHO cells were transfected with siOSBP (25 nM) or siNT for 48 h. Cells were treated with 22OH or 25OH (1.0 μg/ml) with or without RA (10 nM) for 18 h. Whole cell lysates were resolved by SDS-PAGE and immunoblotted using antibodies against ABCA1 and OSBP. D, J774 macrophages were transfected with siNT or siOSBP for 48 h and treated with 22OH (1 μg/ml) or TO901317 (TO) (1 μM) with RA (10 nM) for 18 h prior to immunoblotting for ABCA1, OSBP, and actin.

**Quantification of ABCA1 mRNA**—CHO cells were transfected with siNT or OSBP siRNA for 48 h and treated with 22OH (1 μg/ml) with 10 nM RA, 1 μM TO, or 10 nM RA or solvent for 18 h. Total RNA was purified, and mRNA for ABCA1 and gyceraldehyde-3-phosphate dehydrogenase were quantified by a S1 nuclease protection assay using single-stranded cDNA probes (22). The ABCA1 probe was a 356-bp EcoRI and SacI (nucleotides 2908–2552) fragment of the murine ABCA1 cDNA. ABCA1 expression was quantified by densitometry of autoradiograms using ImageJ 1.38 and expressed relative to gyceraldehyde-3-phosphate dehydrogenase mRNA.

**RESULTS**

**Stable and Transient Gene Silencing of OSBP Increases ABCA1 Expression**—To test whether ABCA1 expression was affected by OSBP depletion, CHO cells stably expressing siRNA reduced OSBP expression by 90% relative to siNT control (28). Compared with siNT-transfected CHO cells, transient knockdown with siOSBP resulted in a consistent increase in ABCA1 expression when cells were treated individually with RA, 22OH, or 25OH and a larger magnitude increase when RA and oxysterols were combined.

Although it was apparent that OSBP depletion induced ABCA1 protein expression following induction with oxysterol agonists of LXR, the effect under basal conditions was difficult to assess due to low and variable ABCA1 expression in CHO cells. To assess whether basal expression was affected by OSBP depletion and if this relationship between OSBP and ABCA1 existed in other cells, ABCA1 expression was measured in J774 macrophages following siRNA transfection (Fig. 1D). ABCA1 expression under basal conditions was readily detected and very responsive to induction by 22OH or TO901317. Transient knockdown of OSBP in untreated J774 macrophages resulted in increased ABCA1 expression (3.0 ± 0.9-fold; mean ± S.E., n = 3) compared with siNT controls. It was also apparent that activation by RA and 22OH or the synthetic LXR agonist TO901317 increased ABCA1 expression in OSBP-depleted cells compared with siNT controls.

The effect of increased ABCA1 expression on cholesterol efflux activity in CHO cells depleted of OSBP was measured by release of [3H]cholesterol to apoA1 in the medium (Fig. 2). siOSBP-transfected cells treated with 22OH or TO901317 and incubated with apoA1 for 1 h had a 3- and 4-fold increase in ABCA1 expression compared with the corresponding non-tar-
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**Figure 2.** siRNA depletion of OSBP increases cholesterol efflux to apoA1. CHO cells were transiently transfected with siOSBP or siNT (25 nm). The following day, cells were loaded with [3H]cholesterol and received no addition (NA), 22OH (1.0 μg/ml) plus 10 μM RA, or 1 μM TO901317 (TO). After 18 h, ABCA1 protein expression and [3H]cholesterol efflux to apoA1 was measured as described under “Experimental Procedures.” A, total lysates from cells treated for 1 h with apoA1 were resolved by SDS-PAGE and immunoblotted for ABCA1, OSBP, and actin. B, ABCA1 protein expression was quantified by densitometry and expressed relative to actin. Results are the mean and S.E. for three separate experiments. C, ApoAI-specific efflux of [3H]cholesterol was measured at 1 h. Results are the mean and S.E. of three separate experiments (*, p < 0.05).

**Figure 3.** Cholesterol extraction by cyclodextrin is not affected by siRNA knockdown of OSBP. CHO cells transiently transfected with siNT or siOSBP (25 nm) for 24 h received medium B with no addition (NA) (●), 22OH (1.0 μg/ml) with 10 nM RA (△), or 1.0 μM TO901317 (■) and were loaded with [3H]cholesterol as described in the legend to Fig. 2. After 18 h, cells were treated with 2 mM MCD in DMEM at 37 °C, and [3H]cholesterol extraction into the medium was quantified at the indicated times. Results are the mean and S.E. of three separate experiments.

**Mechanism** —A possible explanation of the results shown in Figs. 1 and 2 is that OSBP and LXR compete for a common oxysterol ligand(s), and loss of OSBP expression effectively increases the pool of oxysterol available for LXR activation. However, this interpretation appeared to be untenable, since the nonsterol LXR agonist TO901317 increased ABCA1 expression in OSBP-depleted cells to a similar extent as 22OH and 25OH (Figs. 1 and 2). The possibility that OSBP also binds TO901317 was disproved by demonstrating that it failed to compete with 25OH for binding to recombinant OSBP in vitro (supplemental Fig. 1). To determine if OSBP depletion affected LXR activity and ABCA1 gene expression, an S1 nuclease protection assay was used to quantitate ABCA1 mRNA levels in CHO cells transfected with control and OSBP-specific siRNAs (Fig. 4, A and B). Compared with untreated controls, siNT- and siOSBP-transfected CHO cells treated with 22OH or TO901317 displayed a similar 4–5-fold increase in ABCA1 mRNA. The lack of involvement of LXR was further confirmed by showing that luciferase activity driven from a LXR reporter construct was increased 2-fold by 22OH or TO901317 in both siNT- and siOSBP-transfected CHO cells (Fig. 4C).

ABCA1 is also regulated at the post-transcriptional level by calpain dependent proteolysis (11, 12). To test whether OSBP depletion stabilized ABCA1, siNT and siOSBP transfected CHO cells were treated with TO901317, and turnover of ABCA1 protein was monitored by immunoblotting following

Increased apoA1-specific cholesterol efflux in OSBP-depleted cells reflects the increased expression of ABCA1, but underlying changes in cholesterol distribution at the PM or in intracellular compartments could also be involved. Extraction of cellular cholesterol by the nonspecific acceptor methyl-β-cyclodextrin (MCD) is partially dependent on ABCA1 expression (38) but can also be used to assess cholesterol deposition at the PM and internal compartments (39). To determine if cholesterol distribution was affected by OSBP depletion, CHO cells were treated as described in the legend to Fig. 2, and extraction of [3H]cholesterol by MCD in the medium was monitored at 37 °C (Fig. 3). Regardless of OSBP expression and LXR agonist treatment, MCD rapidly absorbed 20–30% of PM [3H]cholesterol by 30 min, after which efflux reached a second slower stage, reflecting mobilization from intracellular sites. Cholesterol extraction from OSBP-depleted cells was slightly reduced but not significantly different from controls, indicating that OSBP does not affect the overall distribution of cholesterol. Treatment of cells with the cholesterol-specific probe BC-θ also confirmed the lack of effect of OSBP-depletion on plasma membrane cholesterol levels (results not shown).

OSBP Regulates ABCA1 Expression by a Post-transcriptional Mechanism —A possible explanation of the results shown in Figs. 1 and 2 is that OSBP and LXR compete for a common oxysterol ligand(s), and loss of OSBP expression effectively increases the pool of oxysterol available for LXR activation. However, this interpretation appeared to be untenable, since the nonsterol LXR agonist TO901317 increased ABCA1 expression in OSBP-depleted cells to a similar extent as 22OH and 25OH (Figs. 1 and 2). The possibility that OSBP also binds TO901317 was disproved by demonstrating that it failed to compete with 25OH for binding to recombinant OSBP in vitro (supplemental Fig. 1). To determine if OSBP depletion affected LXR activity and ABCA1 gene expression, an S1 nuclease protection assay was used to quantitate ABCA1 mRNA levels in CHO cells transfected with control and OSBP-specific siRNAs (Fig. 4, A and B). Compared with untreated controls, siNT- and siOSBP-transfected CHO cells treated with 22OH or TO901317 displayed a similar 4–5-fold increase in ABCA1 mRNA. The lack of involvement of LXR was further confirmed by showing that luciferase activity driven from a LXR reporter construct was increased 2-fold by 22OH or TO901317 in both siNT- and siOSBP-transfected CHO cells (Fig. 4C).

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A

- **siNT**
  - NA
  - 22OH
  - TO

- **siOSBP**
  - NA
  - 22OH
  - TO

**ABCA1**

**GAPDH**

**B**

- **siNT**
  - NA
  - 22OH
  - TO

- **siOSBP**
  - NA
  - 22OH
  - TO

**ABCA1 mRNA expression (relative to GAPDH)**

**C**

- **shNT**
  - NA
  - 22OH
  - TO

- **shOSBP**
  - NA
  - 22OH
  - TO

**Luciferase activity (light units x 10^7)**

**FIGURE 4. Gene silencing of OSBP does not alter ABCA1 mRNA levels or LXR activity.** CHO cells were transiently transfected with siNT or siOSBP (25 nM) duplexes for 24 h. Cells then received medium with no addition (solvent, NA), 22OH (1.0 mM) plus 10 mM RA, or 1.0 μM TO901317 plus RA for 18 h. A and B, total RNA was isolated from cells, and ABCA1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) message levels were quantified by S1 nuclease protection assay and densitometry of autoradiograms. Results are the mean and S.E. of five separate experiments. C, LXR activity was measured using the pGL3-LXREX3 reporter construct as described under "Experimental Procedures." Results are the mean and S.E. of three separate experiments.

inhibition of protein synthesis with cycloheximide (Fig. 5, A and B). ABCA1 in CHO cells transfected with siNT had a short half-life of ~2 h, a value similar to that reported in other cells (9, 14, 41). Depletion of OSBP by siRNA resulted in a significant extension of ABCA1 half-life to 6 h, suggesting that OSBP negatively regulates ABCA1 by protein destabilization. [35S]Methionine/cysteine pulse-chase experiments confirmed that OSBP depletion extended ABCA1 half-life by ~2.5 h (results not shown).

Increased cell surface expression of ABCA1 resulted in stabilization and increased efflux activity (11, 12). To test whether OSBP depletion had a similar effect, the proportion of ABCA1 at the PM was determined by surface biotinylation of siNT- and siOSBP-transfected CHO cells (Fig. 6). As expected, total ABCA1 expression was increased in OSBP-depleted cells treated with or without TO901317. Approximately 20–30% of ABCA1 was biotinylated in untreated siNT and siOSBP-transfected cells. Induction of ABCA1 expression with TO901317 increased the surface presentation of ABCA1 in OSBP-depleted cells; however, the relative fraction at the surface was similar to siNT-treated controls. Unexpectedly, ~20% of total OSBP was associated with the surface biotinylated fraction regardless of ABCA1 or OSBP expression. This “surface-associated” OSBP fraction did not result from direct biotinylation due to cell permeabilization, since other intracellular proteins that are associated with the PM, Golgi, and cytoplasm (protein kinase D and AKT) were not detected.

The OSBP Sterol-binding Domain Is Involved in Negative Regulation of ABCA1—OSBP mediates cholesterol- and 25OH-dependent stimulation of sphingomyelin synthesis by activation of CERT and ceramide delivery to the Golgi apparatus (28). A report that ABCA1-dependent efflux in CHO cells is enhanced by inhibition of sphingomyelin synthesis due to a CERT mutation (42) suggested that a similar mechanism might apply when OSBP expression is silenced. This was examined by treating siNT- and siOSBP-transfected cells with LXR agonists for 18 h and monitoring sphingomyelin synthesis by pulse-labeling with [3H]serine (Fig. 7A) and plasma membrane localization of [3H]choline-labeled SM by bacterial sphingomyelinase treatment (Fig. 7B and C). Similar to previous reports, we observed that stimulation of SM synthesis by 25OH had dissipated by 18 h (43), and 25OH treatment of OSBP-depleted cells caused a 50% reduction in sphingomyelin synthesis (28). However, TO or 22OH had no effect on SM and glucosylceramide synthesis in control or OSBP-depleted CHO cells (Fig. 7A). OSBP-depleted CHO cells labeled to equilibrium with [3H]choline and treated with sphingomyelinase displayed a similar level of plasma membrane sphingomyelin as control cells under all conditions tested. Analysis of total [3H]choline-labeled sphingomyelin in control and sphingomyelinase-treated cells indicated that OSBP depletion reduced sphingomyelin levels in 25OH-treated cells, but 22OH or TO901317 were without affect (Fig. 7C). Thus, there was a lack of correlation between altered sphingomyelin metabolism in response to 25OH and enhanced ABCA1 expression in OSBP-depleted CHO cells.

We previously showed that regulation of SM synthesis by OSBP required the sterol-binding, PH, and FFAT domains (28). If SM synthesis was linked to ABCA1 protein stabilization, then these three domains should be required. To test this and determine which OSBP domain(s) were involved in ABCA1 regulation, CHO cells were co-transfected with a fixed amount of plasmid encoding ABCA1 and increasing amounts of plasmid encoding wild-type OSBP or sterol-binding, PH, and FFAT domain mutants (22, 28) (Fig. 8). Consistent with the stabilizing effect of OSBP depletion, overexpression of OSBP resulted in a 70% decrease in expression of co-transfected ABCA1 (Fig. 8, A and B). A reduction in ABCA1 expression was also observed
when cells were co-transfected with increasing amounts of plasmid encoding OSBP with a PH domain deletion (OSBP-ΔPH) or mutation in the FFAT domain (OSBP-FF/AA), indicating that interaction with phosphatidylinositol 4-phosphate at the Golgi or with VAP in the ER was not required to destabilize ABCA1. In contrast, expression of OSBP-ΔH9004SB, which has a small deletion (ΔH9004432–435) in the sterol-binding domain between the ΔH90251-helical lid and ΔH902521-strand (18) that abrogates 25OH binding (28), did not promote destabilization of ABCA1. Thus, the sterol-binding fold, which is highly conserved in the OSBP gene family, could be important for regulation of ABCA1 protein stability.

DISCUSSION

By virtue of dual organelle-targeting motifs, OSBP partitions between the ER and Golgi apparatus, where it imparts sterol-dependent regulation on ceramide transport and sphingomyelin synthesis. This is an important integrative function, since the relative cholesterol and sphingomyelin content of raft domains controls the activities associated with these specialized membranes (44). Although OSBP appears to be a sterol sensor that is not directly involved in cholesterol regulation in the ER (27, 28), results from this study show that OSBP negatively regulates a key step in the reverse cholesterol transport pathway catalyzed by ABCA1. This suggests that not only does OSBP bind and/or regulate the synthesis of key lipids and sterols destined for efflux; it also regulates one of the ABC proteins that facilitates their transport to extracellular apoA1.

Since OSBP binds a variety of oxysterols and cholesterol, it was surprising that it did not directly impact LXR activity. However, this was clearly the case, since OSBP depletion had no effect on ABCA1 mRNA levels and LXR reporter activity. Instead, ABCA1 protein stability was enhanced 3-fold in the absence of OSBP. It is increasingly apparent that stabilization of the relatively short lived ABCA1 protein (half-life of ~2 h) is an important point of control for cholesterol efflux activity. Increased ABCA1 stability due to association with β1-syntrophin (15) or by deletion of the internal PEST domain (11) is associated with reduced internalization and increased PM association and cholesterol efflux. However, although the absolute amount of ABCA1 was increased in the cell surface fraction of OSBP-depleted cells, the relative proportion of total ABCA1 at the PM relative to controls was not changed. A substantial amount of OSBP was also associated with the surface-biotinylated fraction of cells. However, this fraction of OSBP did not change with ABCA1 expression, and attempts to co-immunoprecipitate ABCA1 and OSBP were unsuccessful (results not shown). Thus, it appears that OSBP affects the exposure of an intracellular pool of ABCA1 to protease degradation by an indi-

![FIGURE 5. RNA interference depletion of OSBP decreases ABCA1 protein turnover. CHO cells were transfected with siNT or siOSBP and treated with TO901317 (1 μM) for 18 h. A, cells received medium containing cycloheximide (CHX) (20 μg/ml) at t = 0 and at the indicated time points were analyzed for expression of ABCA1, OSBP, and actin by immunoblotting. B, ABCA1 protein was quantified by densitometry, normalized to actin, and expressed relative to untreated cells. Results are the mean and S.E. of three or four separate experiments.](https://example.com/figure5)

![FIGURE 6. The proportion of ABCA1 at the plasma membrane is not increased by OSBP depletion. CHO cells were transfected with siNT or siOSBP duplexes (25 nM) for 48 h and received no addition (NA) or 1 μM TO901317 (TO) for 18 h. The proportion of ABCA1, OSBP, protein kinase D (PKD), and AKT in total cell lysates and PM (isolated from 2× total cell lysate) was determined by surface biotinylation and immunoblotting. Similar results were observed in two other experiments.](https://example.com/figure6)
A potential explanation for OSBP-dependent regulation of ABCA1 could involve SM synthesis in the Golgi apparatus. Altering PM sphingomyelin levels by digestion with bacterial sphingomyelinase had variable effects on cholesterol efflux (45, 46). In a related approach, CHO cells with a mutation in sphingomyelinase had variable effects on cholesterol efflux (45, 46). However, the lack of effect of CERT depletion on basal SM synthesis did not influence ABCA1 expression. Our results also suggest that SM metabolism does not contribute to increased ABCA1 protein expression in OSBP-depleted cells. ABCA1 expression was increased by OSBP depletion in control and TO901317- and 22OH-treated cells, even though parameters of sphingomyelin metabolism, such as de novo synthesis, total [3H]choline-labeled pool, and plasma membrane content, were not affected under these conditions. This is consistent with the lack of effect of OSBP depletion on basal SM synthesis (28). On the other hand, activation of SM synthesis by 25OH in sINT transfected cells was significantly reduced by siOSBP depletion (Fig. 7). However, ABCA1 expression was also increased as a consequence of OSBP depletion in 25OH-treated cells (Fig. 1). Thus, OSBP depletion stabilized ABCA1 protein in the absence of measurable changes in SM metabolism and when SM synthesis was altered following 25OH treatment.

A role for sphingomyelin metabolism in ABCA1 protein stability was also supported by co-transfection experiments, which showed that a mutation in the sterol-binding domain, but not the PH or VAP-binding FFAT domains, prevented destabilization of ABCA1 protein. Reconstitution of 25OH-activated sphingomyelin synthesis in OSBP-depleted cells required the sterol-binding, FFAT, and PH domains (28). Although the experimental systems are slightly different, these results support the concept that inhibition of ABCA1 expression by OSBP is independent of its role in sterol-activated sphingomyelin synthesis in the Golgi.
OSBP Negatively Regulates ABCA1 Expression

experiments (Fig. 8) in the absence of exogenous oxysterols, indicating that either 1) an endogenous ligand is sufficient to maximally activate OSBP, thus precluding any effects of exogenous oxysterols, or 2) another region that controls ABCA1 expression was disrupted by the sterol-binding domain mutation.

Since LXR and OSBP have unrelated ligand-binding folds with differing affinities and specificities for oxysterols and cholesterol (18, 51), we propose that the two receptors positively or negatively regulate ABCA1 expression in response to different signals. Excess cholesterol could generate a pool of oxysterols that specifically activate LXR in the nucleus and increase expression of the ABCA1 gene. By contrast, OSBP could be activated by cytoplasmic oxysterols or cholesterol as a means of fine tuning the expression of ABCA1 in specific compartments of the secretory pathway. Negative regulation of ABCA1 expression by OSBP and ORP8 (36), albeit by different mechanisms, identifies these receptors as potential therapeutic targets to enhance cholesterol efflux from sensitive tissues.

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