Molecular Biology of the Cell
Vol. 17, 2604–2616, June 2006

# Oxysterol-binding Protein and Vesicle-associated Membrane Protein–associated Protein Are Required for Sterol-dependent Activation of the Ceramide Transport Protein[D]

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Submitted January 23, 2006; Revised March 2, 2006; Accepted March 22, 2006
Monitoring Editor: Sean Munro

**Sphingomyelin (SM) and cholesterol are coregulated metabolically and associate physically in membrane microdomains involved in cargo sorting and signaling. One mechanism for regulation of this metabolic interface involves oxysterol binding protein (OSBP) via high-affinity binding to oxysterol regulators of cholesterol homeostasis and activation of SM synthesis at the Golgi apparatus. Here, we show that OSBP regulation of SM synthesis involves the endoplasmic reticulum (ER)-to-Golgi ceramide transport protein (CERT). RNA interference (RNAi) experiments in Chinese hamster ovary (CHO)-K1 cells revealed that OSBP and vesicle-associated membrane protein–associated protein (VAP) were required for stimulation of CERT-dependent ceramide transport and SM synthesis by 25-hydroxycholesterol and cholesterol depletion in response to cyclodextrin. Additional RNAi experiments in human embryonic kidney 293 cells supported OSBP involvement in oxysterol-activated SM synthesis and also revealed a role for OSBP in basal SM synthesis. Activation of ER-to-Golgi ceramide transport in CHO-K1 cells required interaction of OSBP with the ER and Golgi apparatus, OSBP-dependent Golgi translocation of CERT, and enhanced CERT–VAP interaction. Regulation of CERT by OSBP, sterols, and VAP reveals a novel mechanism for integrating sterol regulatory signals with ceramide transport and SM synthesis in the Golgi apparatus.**

## INTRODUCTION

Oxysterols comprise a large group of hydroxy, keto, or epoxy derivatives of cholesterol produced by hydroxylases (Russell, 2000) or during de novo cholesterol synthesis (Nelson *et al*., 1981; Rowe *et al*., 2003). Oxysterols are primarily involved in transcriptional and posttranscriptional regulation of cholesterol metabolism via the liver X receptor (Janowski *et al*., 1996; Venkateswaran *et al*., 2000), proteolytic activation of the sterol regulatory element binding protein transcription factors (Adams *et al*., 2003; Radhakrishnan *et al*., 2004), and degradation of HMG-CoA reductase (Song and DeBose-Boyd, 2004).

In addition to interacting directly with components of the cholesterol regulatory machinery, oxysterols are also bound by the oxysterol binding protein (OSBP) and OSBP-related protein (ORP) 4 (Ridgway *et al*., 1992; Wang *et al*., 2002), a subgroup within the 12-member mammalian ORP gene family (Lehto *et al*., 2001). A homologous family of seven proteins (Osh1p-Osh7p) has also been identified in the budding yeast *Saccharomyces cerevisiae* (Beh *et al*., 2001). The ORP family is characterized by a highly conserved 350 amino acid C-terminal sterol or phospholipid binding domain (Ridgway *et al*., 1992; Xu *et al*., 2001; Li *et al*., 2002) and an N-terminal pleckstrin homology (PH) domain. Crystallographic analysis of the Osh4p sterol binding revealed a 19-strand $\beta$-barrel, which accommodated both sterol and oxysterol ligands in a hydrophobic interior, capped with a flexible N-terminal lid (Im *et al*., 2005). This suggested a role in sterol transport between membranes or mediation of sterol-dependent signaling. Adjacent to the oxysterol binding domain in 11 OSH/ORPs is a FFAT (two phenylalanines in an acidic tract) motif that binds vesicle-associated membrane protein–associated protein-A (VAP-A) or the yeast orthologue Scs2 in the endoplasmic reticulum (ER) (Wyles *et al*., 2002; Loewen *et al*., 2003; Wyles and Ridgway, 2004; Loewen and Levine, 2005). The FFAT motif is also present in other lipid binding/regulatory proteins (Nir1-3, Opi1p, and CERT), indicating that ER recruitment is a conserved feature of the lipid transport or regulatory functions of these proteins (Loewen *et al*., 2004; Loewen and Levine, 2005). OSBP localizes to the cytoplasm or with VAP in the ER (Wyles *et al*., 2002), but it translocates to the Golgi apparatus when cells are exposed to 25-hydroxycholesterol (25OH) or conditions that deplete cellular cholesterol and/or activate de novo cholesterol synthesis (Ridgway *et al*., 1992, 1998; Mohammadi *et al*., 2001). Localization of OSBP to the Golgi apparatus in response to oxysterols involves binding of phosphatidylinositol-4-phosphate (PtdIns-4-P) and Arf1 by

This article was published online ahead of print in *MBC in Press* (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E06–01–0060) on March 29, 2006.

[D] The online version of this article contains supplemental material at *MBC Online* (http://www.molbiolcell.org).

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Abbreviations used: $C_5$-DMB-ceramide, *N*-[5-(5,7dimethyl boron dipyrromethenedifluoride)-1-pentanoyl]-D-sphingosine; CERT, ceramide transport protein; FFAT, two phenylalanines in an acidic tract; 25OH, 25-hydroxycholesterol; GlcCer, glucosylceramide; M$\beta$CD, methyl-$\beta$-cyclodextrin; NT, nontargeting; PtdEtn, phosphatidylethanolamine; PtdSer, phosphatidylserine; PtdIns-4-P, phosphatidylinositol-4-phosphate; OSBP, oxysterol binding protein; ORP, OSBP-related protein; RNAi, RNA interference; shRNA, short hairpin RNA; siRNA, short interfering RNA; SM, sphingomyelin; VAP, vesicle-associated membrane protein-associated protein.

the PH domain (Lagace *et al*., 1997; Levine and Munro, 1998, 2002; Godi *et al*., 2004).

Overexpression of OSBP or ORP4 in Chinese hamster ovary (CHO)-K1 cells caused a modest enhancement of basal cholesterol synthesis but did not affect the suppression of cholesterol synthesis by 25OH (Lagace *et al*., 1997; Wang *et al*., 2002). Overexpression of other mammalian ORPs (Laitinen *et al*., 2002; Johansson *et al*., 2003) or deletion of OSH genes in yeast (Beh *et al*., 2001; Beh and Rine, 2004) caused pleiotropic effects on sterol/lipid metabolism and trafficking, but it did not reveal specific modes of action or biological targets for these proteins. These variable and minor effects on sterol metabolism could be due to functional redundancy within the family (Beh *et al*., 2001) or to a secondary response to the primary function of OSBP or ORPs, such as sterol transport or integration of sterol sensing with other metabolic functions. In support of the latter function, Osh4p binds a variety of sterols and oxysterols (Im *et al*., 2005) and also negatively regulates Golgi vesicle biogenesis mediated by the phosphatidylinositol/phosphatidylcholine transfer protein Sec14p (Fang *et al*., 1996). Additionally, OSBP serves as a cholesterol-sensitive scaffold protein for two phosphatases that control the activity of extracellular signal-regulated kinase 1/2 signaling pathways (Wang *et al*., 2005) and has an oxysterol-sensing function with regard to regulation of sphingomyelin (SM) metabolism. Exposure of CHO-K1 cells to exogenous 25OH elicited a threefold stimulation of SM synthesis (Ridgway, 1995), the result of increased delivery of ceramide to SM synthase in the Golgi apparatus (Huitema *et al*., 2004). Stimulation of SM synthesis by 25OH was further enhanced by overexpression of OSBP (Lagace *et al*., 1999) and inhibited by expression of a PH domain mutant of OSBP that constitutively localized with VAP in the ER (Wyles *et al*., 2002). This suggested that 25OH-dependent translocation of OSBP to the Golgi apparatus activated a ceramide transport pathway specific for SM synthesis. This could be a mechanism to maintain stoichiometry between SM and cholesterol whereby oxysterols, produced as a consequence of increased cholesterol uptake or elevated de novo synthesis, signal through OSBP to increase the rate of SM synthesis accordingly.

The mechanism of ER-to-Golgi ceramide transport was ambiguous until Hanada and coworkers cloned the cDNA for a ceramide transport protein (CERT) that mediated monomeric lipid transfer between the ER and Golgi apparatus (Fukasawa *et al*., 1999; Hanada *et al*., 2003). CERT contains a C-terminal steroidogenic acute regulatory-related lipid transfer domain that binds ceramide and an N-terminal PH domain that specifically binds PtdIns-4-P at the Golgi apparatus (Hanada *et al*., 2003; Kumagai *et al*., 2005). In addition, CERT has a FFAT motif positioned between the PH and steroidogenic acute regulatory-related lipid transfer domains (Loewen *et al*., 2003). This suggests that OSBP could regulate CERT activity by directly or indirectly interacting with shared binding partners at the Golgi apparatus and/or ER. To address this hypothesis, we used RNA interference (RNAi) to show that OSBP and VAP are required for basal and sterol-dependent CERT activity and SM synthesis. On a mechanistic level, this involved OSBP-dependent translocation of CERT to the Golgi apparatus along with enhanced interaction of a CERT subfraction with the common ER binding partner VAP, suggesting dynamic movement of CERT between these two organelles. This identifies a novel interaction between two structurally related lipid binding proteins that serves to mediate sterol-dependent integration of SM and cholesterol biosynthetic pathways.

## MATERIALS AND METHODS

### *Plasmids, Antibodies, and Cell Culture*

The human CERT cDNA was amplified by PCR from pCMV6-CERT (Origene Technologies, Rockville, MD) and cloned into pcDNA3.1-V5/his, pEGFP-N1, pACT2, and pAS1. pcDNA-CERT-FF/AA and pCERT-GFP-FF/AA encode CERT with a mutagenized FFAT motif ($FF^{321,322}AA$). pOSBP resistant (resOSBP) to targeting by an OSBP-specific short-hairpin RNA (shRNA) was made by mutagenesis at nucleotide (nt) 1216 (a→c), 1218 (a→t), and 1221 (c→g). pACT2-VAP-A, pACT2-OSBP, pAS1-VAP-A, and pAS1-OSBP were described previously (Wyles *et al*., 2002). A CERT polyclonal IgY was from Genway Biotech (San Diego, CA). Antibodies against giantin, ORP4, OSBP, and VAP were described previously (Ridgway *et al*., 1992, 1998; Mohammadi *et al*., 2001; Wang *et al*., 2002; Wyles *et al*., 2002). Human embryonic kidney (HEK) 293 and COS-7 cells were cultured in DMEM containing 10% (vol/vol) fetal calf serum (FCS) (medium A). CHO-K1 cells were maintained in DMEM containing 5% (vol/vol) FCS and 33 μg/ml proline (medium B).

### *RNAi Reagents and Cell Transfection*

Potential RNAi target sites in human and hamster OSBP were identified (Elbashir *et al*., 2002) and used to construct pSuppressor vectors (Imgenex, San Diego, CA) encoding shRNAs. A pSuppressor vector for OSBP (shOSBP), identified by transient transfected into CHO-K1 cells and corresponding to nt 1215-1233 (5′-gagaaccagaataccatac-3′) of the human cDNA, afforded >50% knockdown of OSBP protein expression. The nontargeting (NT) shRNA vector (shNT) contained a g→t mutation at nt 1217. CHO-K1 cell clones with constitutive knockdown of OSBP expression were generated by calcium phosphate transfection with shOSBP (controls were transfected with shNT), selected in medium B containing 500 μg/ml G418 for 2 wk, and screened for OSBP protein expression by immunoblotting. Two independent clones (shOSBP1 and shOSBP2) had similar properties (Supplemental Figure 1), and shOSBP1 was used for experiments unless stated otherwise.

Short interfering RNA (siRNA) duplexes for VAP, CERT, OSBP, and NT controls (siNT) were purchased from Dharmacon (Lafayette, CO) and transiently transfected into HEK293 or CHO-K1 cells with TransIT-TKO (Mirus, Madison, WI) in serum-containing medium for 36–48 h. HEK293 cells were transfected with 75 nM siOSBP1, 25 nM each of siCERT2 and siCERT4, and/or 125 nM siNT. For transient knockdown of CERT and VAP, shNT or shOSBP cells were transfected with 25 nM siCERT2 and/or 25 nM siVAP3 (50 nM siNT as a control). Transient knockdown in CHO-K1 cells used 25 nM each of siCERT2 and/or siOSBP2 (50 nM of siNT as a control).

### *Incorporation of Radioactive Precursors into Sphingolipids, Phospholipids, and Cholesterol*

Sphingolipids and phospholipids in HEK293 and CHO-K1 cells were labeled with 20 and 10 μCi/ml [$^3$H]serine, respectively, for the final 2 h of a 6-h treatment with 6 μM 25OH (Steraloids, Newport, RI) in serine-free medium. For methyl-β-cyclodextrin (MβCD) experiments, cells were treated for 1 h with 5 mM MβCD in serine-free DMEM containing 5% lipoprotein-deficient serum (medium C) and incubated with 10 μCi/ml [$^3$H]serine in medium C for 6 h. [$^3$H]Serine incorporation into sphingolipids and phospholipids was quantified after lipid extraction and thin layer chromatography as described previously (Perry and Ridgway, 2004). Cholesterol and fatty acid synthesis was assessed in cells cultured in medium C for 16–20 h before addition of 6 μM 25OH for 2 h and incubation with 5 μCi/ml [$^{14}$C]acetate for a further 2 h (Lagace *et al*., 2000).

### *Coimmunoprecipitation and Glutathione* S*-Transferase (GST) Pull-Downs*

CHO-K1 cells in monolayer were treated with 1 mM dithiobis succinimidyl propionate (Pierce Chemical, Rockford, IL) in phosphate-buffered saline at 20°C. The reaction was terminated by adding cold Tris-buffered saline (25 mM Tris-HCl, pH 7.4, 140 mM NaCl, 3 mM KCl, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 0.9 mM $Na_2PO_4$), and cells were harvested and solubilized on ice in lysis buffer A (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 60 mM *n*-octyl β-D-glucopyranoside, 1 mM EDTA, 1 mM EGTA, and a protease inhibitor cocktail). Supernatants were prepared by centrifugation at 16,000 × *g* for 15 min at 4°C and incubated with 2 μl of OSBP, VAP-A, or nonimmune polyclonal antibodies overnight at 4°C followed by protein A-Sepharose for 30 min at 20°C. Sepharose beads were collected by centrifugation, and cross-linked proteins were cleaved and denatured by addition of 5× SDS sample buffer under reducing conditions, separated by SDS-PAGE, and analyzed by immunoblotting.

OSBP and CERT cDNAs were transiently transfected into COS-7 cells by the DEAE-dextran method (Esser *et al*., 1988). OSBP and CERT in 30 μl of Triton X-100 extracts from transfected cells were analyzed for interaction with GST or GST-VAP-A (Wyles *et al*., 2002).

### *Yeast Two-Hybrid Interaction*

Yeast two-hybrid vectors were transformed into strain PJ69 (ATCC 201450), and detection of interactions between OSBP, VAP-A, and CERT were determined by growth on selective plates (Wyles *et al*., 2002).

### *Immunofluorescence Microscopy*

Cells were processed for immunofluorescence analysis as described previously (Ridgway *et al*., 1992). Cells expressing CERT-GFP were fixed in 4% paraformaldehyde and mounted with Mowiol 4-88 reagent (Calbiochem, La Jolla, CA). Cells were viewed and images were captured using a Zeiss LSM 510/AxioVert 100M inverted microscope and a plan-apochromat 100×/1.40 oil immersion objective. Images were prepared using Zeiss LSM Image Viewer and Adobe Photoshop software (Adobe Systems, Mountain View, CA).

The transport of *N*-[5-(5,7-dimethyl boron dipyrromethene difluoride)-1-pentanoyl]-D-sphingosine ($C_5$-DMB-ceramide) from the ER to Golgi apparatus in intact CHO-K1 cells treated for 2 h with 6 $\mu$M 25OH in medium B was monitored as described previously (Perry and Ridgway, 2004). $C_5$-DMB-ceramide localization was viewed and photographed using a Zeiss AxioVert 200m inverted microscope fitted with an AxioCam HRm, FluorArc fluorescence source, and plan-neofluor 100×/1.3 oil immersion objective. Final images were prepared using Adobe Photoshop software.

## RESULTS

### *OSBP and 25OH Activate CERT-dependent Ceramide Transport and SM Synthesis*

To investigate the role of OSBP in ceramide transport and SM synthesis, OSBP expression in CHO-K1 cells was suppressed by stable expression of a shRNA specific for OSBP (shOSBP) or a nontargeting control (shNT) (Figure 1, A and B). CERT expression was reduced alone or in combination with OSBP depletion by transient transfection of an siRNA duplex into CHO-K1 cells expressing shNT or shOSBP, respectively. Figure 1A shows a representative immunoblot of CERT, OSBP, ORP4, and VAP expression in extracts from OSBP and CERT knockdown cells, and quantification of expression relative to actin (Figure 1B). shRNA or siRNA methods resulted in 80–90% suppression of OSBP and CERT protein expression without affecting the OSBP/ORP partner-protein VAP or ORP4.

To determine whether the consequences of OSBP and CERT depletion were applicable to other cell lines, a parallel series of experiments were performed in which OSBP and CERT expression was knocked down by transient siRNA transfection in HEK293 cells (Figure 1, C and D). Immunoblot analysis (Figure 1C) and quantification (Figure 1D) of CERT and OSBP in siRNA-transfected HEK293 cells revealed a 75% reduction in protein expression, slightly less than that achieved in CHO-K1 cells (Figure 1, A and B).

SM synthesis in CHO-K1 cells is dependent on CERT transport of ceramide from its site of synthesis in the ER to the Golgi apparatus (Hanada *et al*., 2003). This transport pathway can be conveniently monitored in cells by measuring incorporation of [$^3$H]serine into ceramide and SM during a 2-h labeling period. At the same time, [$^3$H]serine incorporation into glucosylceramide (GlcCer), phosphatidylserine (PtdSer), and phosphatidylethanolamine (PtdEtn) can be used as an indicator of global or nonspecific effects of RNAi on isotope uptake and lipid synthesis. CHO-K1 cells, in which OSBP or CERT expression was suppressed by RNAi, were cultured in the presence or absence of 25OH, pulse-labeled with [$^3$H]serine and incorporation of the label into ceramide, SM, GlcCer, PtdSer, and PtdEtn was quantified (Figure 2, A and B). Consistent with previous studies (Ridgway, 1995; Lagace *et al*., 1997), control CHO-K1 cells treated with 25OH displayed a twofold increase in [$^3$H]serine incorporation into SM but not GlcCer or ceramide (Figure 2A). Decreased OSBP expression had no affect on basal SM synthesis, but it completely blocked the 25OH-dependent stimulation of SM synthesis. This loss of response to 25OH in

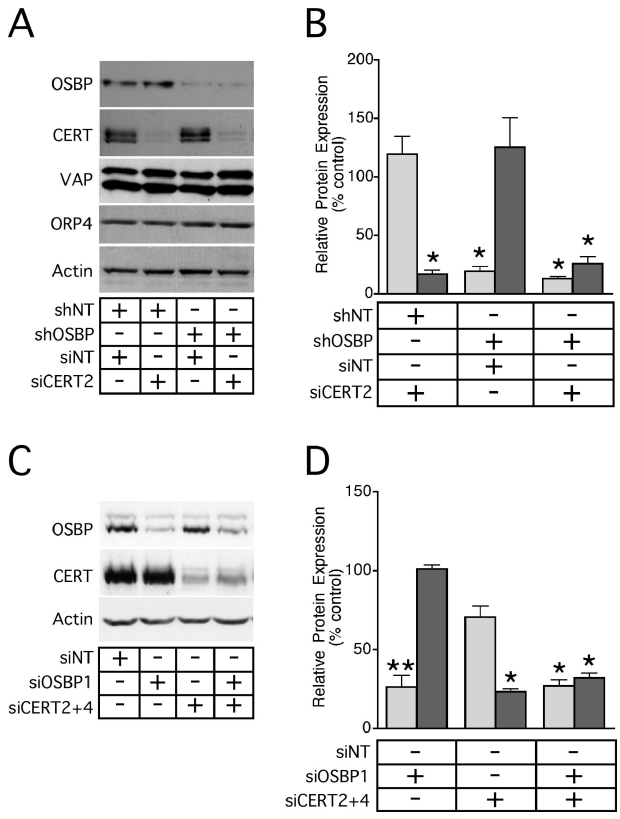


**Figure 1.** Suppression of OSBP and CERT protein expression by RNAi. The expression of OSBP and/or CERT was suppressed in CHO-K1 (A and B) or HEK293 (C and D) by transient transfection of siRNAs or stable expression of shRNAs as described in *Materials and Methods*. Control cells were transfected with NT siRNAs or shRNAs. Whole cells lysates from CHO-K1 (A) and HEK293 (C) cells were resolved by SDS-10% PAGE, transferred to nitrocellulose, and immunoblotted for CERT, OSBP, VAP, ORP4, or actin. OSBP (light gray bars) and CERT (dark gray bars) suppression in CHO-K1 (B) and HEK293 (D) cells relative to control cells was quantified by densitometry using actin as a load control. Results are mean and SE of three to five experiments. *$p \leq 0.005$ and **$p \leq 0.01$.

OSBP-depleted cells was confirmed in another independently isolated cell line stably expressing shOSBP and treated with a 0–6 $\mu$M 25OH (Supplemental Figure 1). siRNA suppression of CERT reduced basal SM synthesis by 50% and blocked the 25OH-mediated increase in SM synthesis, but it had no effect on GlcCer (Figure 2A), consistent with a specific role in ceramide delivery to SM synthase 1 in the Golgi apparatus (Hanada *et al*., 2003; Huitema *et al*., 2004). siRNA suppression of CERT also caused a minor decrease in [$^3$H]serine incorporation into ceramide, consistent with results using CERT-defective LY-A cells (Fukasawa *et al*., 1999). Interestingly, simultaneous suppression of CERT and OSBP expression did not further attenuate basal SM synthesis, but 25OH treatment caused inhibition of SM synthesis by a further 60%. In this instance, suppression of SM synthesis was traced to a significant 40% reduction in [$^3$H]serine incorporation into ceramide (Figure 2A). This suggests that not only is OSBP involved in CERT-dependent ceramide transport but also that it seems to regulate ceramide metabolism in the ER. Reduction in CERT and/or OSBP had no effect on PtdSer or PtdEtn synthesis (Figure 2B), confirming that the observed effects are specific for the SM biosynthetic pathway. As well, the results shown in Figure 2A were not

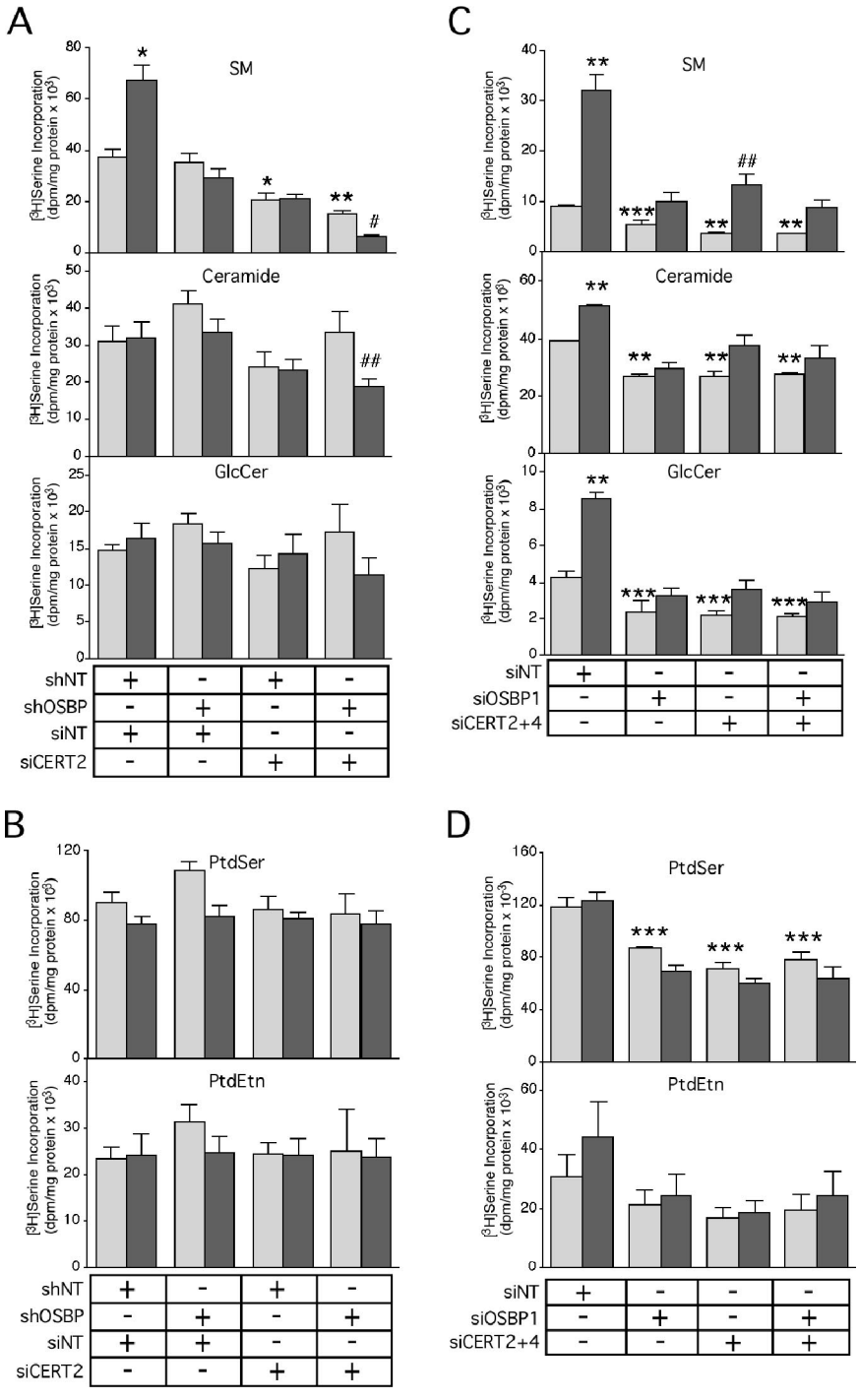


**Figure 2.** Effect of RNAi suppression of OSBP and CERT on SM synthesis in CHO-K1 and HEK293 cells. Sphingolipid, PtdEtn, and PtdSer synthesis was measured by [$^3$H]serine incorporation in shNT and shOSBP cells transfected with NT or CERT-specific siRNA as described in *Materials and Methods* (A and B). Cells were treated with 25OH (6 $\mu$M, dark gray bars) or solvent control (light gray bars) for 4 h followed by incubation with [$^3$H]serine for an additional 2 h. After 6 h, cells were harvested and [$^3$H]serine incorporation into SM, ceramide, and GlcCer (A) or PtdEtn and PtdSer (B) was quantified. HEK293 cells were transiently transfected with NT, OSBP, and/or CERT siRNAs and synthesis of SM, ceramide, and GlcCer (C) or PtdEtn and PtdSer (D) was measured by [$^3$H]serine incorporation in cells treated with 25OH (dark gray bars) or solvent (light gray bars) as described above. Results are the mean and SE from three or four experiments (A, C, and D) or mean and range for two experiments (B). *$p \leq 0.005$, **$p \leq 0.001$, and ***$p \leq 0.02$ compared with solvent-treated shNT/siNT or siNT-transfected cells. $^{\#}p \leq 0.001$ and $^{\#\#}p \leq 0.02$, compared with paired solvent-treated cells.

an adaptive response to stable suppression of OSBP, because similar effects on SM synthesis were observed in CHO-K1 cells in which both OSBP and CERT expression was suppressed by transient transfection of siRNAs for 48 h (Supplemental Figure 2).

Analysis of SM biosynthesis was performed in HEK293 cells transiently knocked down for OSBP and/or CERT to determine whether these proteins were involved in 25OH-dependent ceramide transport in other cell types (Figure 2, C and D). Control siNT-transfected HEK293 cells displayed a robust 3.5-fold increase in SM synthesis when exposed to 25OH for 6 h (Figure 2C). Reduced OSBP expression abolished the response to 25OH but, unlike results in CHO-K1 cells, it also caused a significant 40% reduction in basal SM synthesis. Depletion of CERT caused basal and 25OH-stimulated SM synthesis rates to both decline by 70%, but the 3.5-fold increase in SM synthesis in response to 25OH was maintained. Suppression of both CERT and OSBP did not further inhibit basal SM synthesis, and stimulation of SM synthesis in response to 25OH was no longer significant. In contrast to CHO-K1 cells, 25OH caused a twofold increase in GlcCer synthesis that was suppressed by knockdown of OSBP or CERT expression. PtdSer and PtdEtn synthesis was reduced 10–50% in OSBP or CERT siRNA-transfected cells

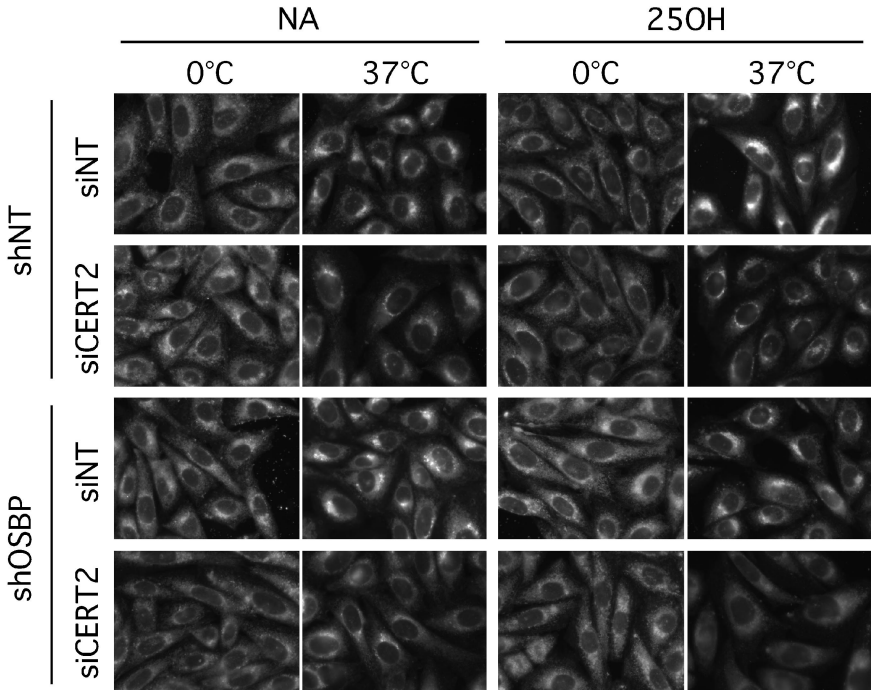


**Figure 3.** $C_5$-DMB-ceramide transport to the Golgi apparatus in OSBP and CERT depleted CHO-K1 cells. CHO-K1 cells depleted of OSBP and/or CERT by RNAi were pretreated with 6 $\mu$M 25OH or solvent (NA) followed by a 30-min incubation in serum-free media DMEM containing 1 $\mu$M $C_5$-DMB-ceramide (complexed to bovine serum albumin [BSA]) at 4°C. Cells then received fresh DMEM containing 0.34 mg/ml BSA without $C_5$-DMB-ceramide at 37°C for 18 min and were processed for microscopy. The results are representative of three independent experiments.

(Figure 2D). Although knockdown of OSBP prevented stimulation of SM synthesis in both CHO-K1 and HEK293, there were obvious differences in the response of these cells to OSBP and CERT depletion. We attributed these differences to two factors: 1) a reduced rate of sphingolipid synthesis in HEK293 cells (basal SM synthesis in CHO-K1 cells was 4-fold higher) and 2) weaker suppression of OSBP and CERT expression by RNAi in HEK293 cells (Figure 1). Taking these factors into account, as well as the pronounced effects of RNAi and 25OH on GlcCer, PtdSer, and PtdEtn synthesis in HEK293 cells (Figure 2, A and B), further experiments were preformed in CHO-K1 cells.

Overexpression of OSBP in CHO-K1 cells increased basal cholesterol synthesis, but it did not affect suppression by 25OH (Lagace *et al*., 1997). RNAi depletion of OSBP in HeLa cells did not affect basal or 25OH-dependent cholesterol regulation (Nishimura *et al*., 2005), a result we have confirmed in CHO-K1 cells (Supplemental Figure 3). OSBP depletion in CHO-K1 cells had no affect on cholesterol or fatty acid synthesis either in the presence or absence of 25OH. Similarly, CERT depletion did not significantly affect cholesterol synthesis, but there was a trend for increased [$^{14}$C]acetate incorporation into fatty acids. These metabolic labeling experiments demonstrate that OSBP involvement in 25OH-dependent activation of CERT-dependent ceramide transport and SM synthesis is independent of changes in cholesterol synthesis.

In the absence of any indication of [$^3$H]ceramide accumulation in the ER of OSBP- or CERT-depleted CHO-K1 or HEK293 cells (Figure 2), we opted to directly monitor the effect of OSBP and 25OH on CERT-dependent ceramide transport from the ER-to-Golgi apparatus using the fluorescent ceramide analogue $C_5$-DMB-ceramide (Pagano *et al*., 1991; Fukasawa *et al*., 1999; Hanada *et al*., 2003). After RNAi to reduce OSBP and/or CERT expression and exposure to 25OH, CHO-K1 cells were loaded with $C_5$-DMB-ceramide at 4°C then shifted to 37°C to facilitate transfer to the Golgi apparatus (Figure 3). Under all conditions, $C_5$-DMB-ceramide was clearly visible in the ER after incubation at 4°C. In control cells shifted to 37°C, $C_5$-DMB-ceramide was localized to a perinuclear region corresponding to the Golgi apparatus (Pagano *et al*., 1991). This was enhanced in cells treated with 25OH for 2 h before loading with $C_5$-DMB-ceramide. In cells with reduced CERT expression, transfer of $C_5$-DMB-ceramide to the Golgi apparatus was substantially diminished in the both the presence and absence of 25OH. Under basal conditions (no addition, NA), $C_5$-DMB-ceramide transport to the Golgi apparatus at 37°C in OSBP-depleted cells was similar to that in controls, but increased Golgi-associated fluorescence in response to 25OH was not evident. It is notable that in 25OH-treated OSBP/CERT knockdown cells, there was a further diminution in Golgi-associated $C_5$-DMB-ceramide after incubation at 37°C. To assess whether this was due to fragmentation of the Golgi apparatus by CERT and OSBP depletion, organization of the Golgi apparatus was assessed by immunostaining with the vesicle tethering protein giantin (Supplemental Figure 4). Although suppression of OSBP expression did not affect the localization of giantin, CERT knockdown caused a slight but reproducible fragmentation of Golgi-localized giantin, in the presence or absence of 25OH, that was also observed when immunostaining for OSBP- in CERT-depleted CHO-K1 cells (Figure 5C). Because the Golgi apparatus is intact, it seems that in the absence of $C_5$-DMB-ceramide transport to the Golgi apparatus in 25OH-treated CERT- and OSBP-depleted cells, $C_5$-DMB-ceramide could be metabolized or exported from the cell, leading to an overall reduction in fluorescence.

Because OSBP was reported to bind cholesterol (Wang *et al*., 2005), it could also mediate changes in SM synthesis in response to alterations in cellular cholesterol levels (Ridgway, 2000). We investigated this by determining whether OSBP was required for stimulation of SM synthesis at the Golgi apparatus after M$\beta$CD depletion of cellular cholesterol (Leppimaki *et al*., 1998). SM synthesis was measured by [$^3$H]serine incorporation in CHO-K1 cells depleted of OSBP or CERT by RNAi and exposed to 5 mM M$\beta$CD for 1 h (Figure 4). M$\beta$CD removed 60% of a [$^3$H]cholesterol tracer from both shNT and shOSBP cells after 1 h (our unpublished data). Control cells trans-

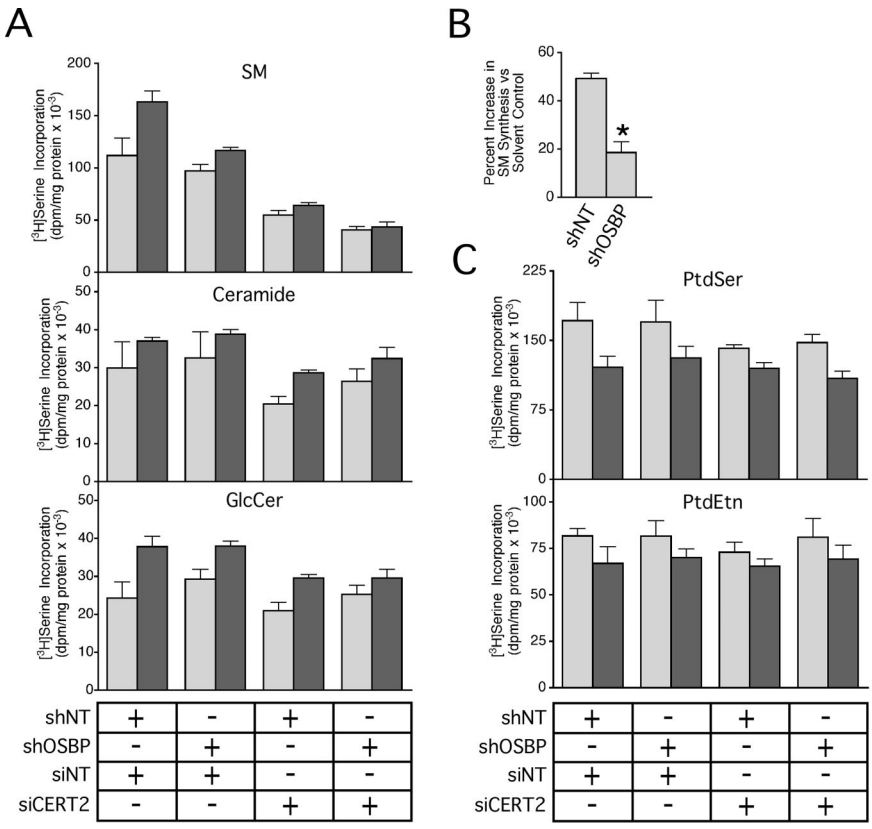


**Figure 4.** Activation of SM synthesis by cholesterol depletion is dependent on OSBP. shNT and shOSBP cells were transiently transfected with NT or CERT-specific siRNA, treated with 5 mM M$\beta$CD (dark gray bars) or solvent (light gray bars) for 1 h, and incubated with 10 $\mu$Ci/ml [$^3$H]serine for 6 h. Cells were harvested and isotope incorporation into SM, ceramide, and GlcCer (A) or PtdEtn and PtdSer (C) was quantified. Results are the mean and SD of a single experiment performed in triplicate and repeated twice with similar results. (B) Percentage of increase in SM synthesis in shNT and shOSBP cells treated with M$\beta$CD compared with solvent controls from three independent experiments. *$p \leq 0.005$ compared with shNT cells.

fected with shNT and siNT and exposed to M$\beta$CD had a 50% increase in [$^3$H]serine incorporation into SM and GlcCer, respectively (Figure 4, A and B). Depletion of OSBP did not affect basal SM synthesis but partially prevented the increase in SM synthesis in response to M$\beta$CD (Figure 4, A and B). Depletion of CERT or CERT/OSBP reduced basal SM synthesis by 50–60% and blocked the increase in SM synthesis in response to M$\beta$CD (Figure 4A). Synthesis of PtdSer and PtdEtn were not affected by RNAi or M$\beta$CD (Figure 4B). Thus, OSBP also activates ceramide transport and SM synthesis in response to depletion of cellular cholesterol.

### *OSBP and 25OH Increase Golgi Localization of CERT*

Because OSBP undergoes 25OH-mediated translocation to the Golgi apparatus (Ridgway *et al.*, 1992; Lagace *et al.*, 1997), increased ceramide transport could involve OSBP-facilitated CERT translocation to that organelle. This was examined using indirect immunofluorescence microscopy of endogenous OSBP and CERT in control and 25OH-treated CHO-K1 cells (Figure 5). In untreated cells expressing shNT, both OSBP and CERT were diffusely localized throughout the cell with minimal colocalization at perinuclear Golgi structures (Figure 5A). Treatment of cells with 25OH for 2 h caused translocation and colocalization of both CERT and OSBP at the Golgi apparatus. To determine whether OSBP was required for CERT translocation to the Golgi apparatus, CERT localization was examined in CHO-K1 cells expressing shOSBP (Figure 5A). OSBP depletion did not affect the distribution of CERT under control conditions, but it prevented 25OH-mediated CERT translocation to the Golgi apparatus. Similar to endogenous CERT, Golgi localization of CERT-GFP in shNT cells was increased in the presence of 25OH but prevented if OSBP was depleted (Figure 5B). In a complimentary experiment, CERT depletion did not affect the 25OH-dependent localization of OSBP to the Golgi apparatus (Figure 5C). However, the pattern of OSBP localization at the Golgi apparatus was slightly fragmented in CERT-depleted cells.

Although there was strong colocalization of CERT and OSBP at punctate perinuclear structures in 25OH-treated CHO-K1 cells, their common ER binding partner VAP did not show a similar pattern of localization (Supplemental Figure 5). However, VAP was present at diffuse perinuclear sites under control conditions, which was enhanced by 25OH treatment resulting in partial overlap with CERT immunofluorescence.

Immunofluorescence experiments were also performed to determine whether M$\beta$CD-mediated stimulation of SM synthesis by OSBP involved enhanced localization of CERT to the Golgi apparatus (Figure 6). M$\beta$CD treatment of shNT expressing cells resulted in extensive colocalization of OSBP and CERT at the Golgi apparatus. However, CERT remained diffusely distributed throughout shOSBP cells treated with M$\beta$CD. Thus, localization of CERT at the Golgi apparatus was OSBP-dependent and correlated with increased SM synthesis and ceramide transport in two independent experimental models.

### *OSBP Interaction with the ER and Golgi Is Required to Enhance CERT Activity*

To assess which OSBP domains were required for stimulation of CERT activity, OSBP-depleted CHO-K1 cells were transiently transfected with shRNA resistant OSBP (resOSBP), resOSBP $\Delta$PH (PH domain deletion), resOSBP $\Delta$432-435 (oxysterol binding defective), and resOSBP FF/AA (FFAT mutation)

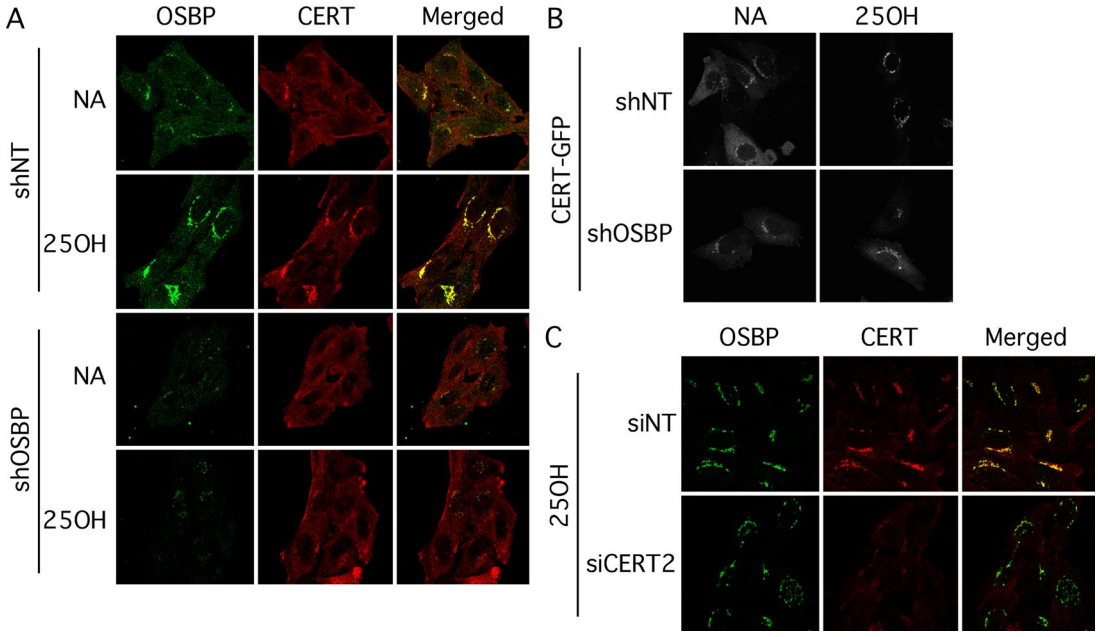


**Figure 5.** OSBP and 25OH promote CERT translocation to the Golgi apparatus. (A) Endogenous OSBP and CERT were detected in shNT and shOSBP cells after incubation with 25OH or solvent (NA) for 2 h as described in *Materials and Methods*. OSBP was detected with a primary polyclonal antibody and an Alexa Fluor 488-conjugated goat anti-rabbit secondary antibody. CERT was detected with an IgY antibody followed by an Alexa Fluor 594-conjugated goat anti-chicken secondary antibody. (B) shNT or shOSBP cells were transiently transfected with pCERT-GFP and treated with 6 $\mu$M 25OH or NA for 2 h. (C) shNT cells were transfected with siNT or a CERT-specific siRNA, and the localization of CERT and OSBP was determined after 25OH treatment as described in A. Confocal images are single optical sections of 1.6 $\mu$m.

(Figure 7). 25OH-stimulated SM synthesis was restored by expression of resOSBP in OSBP-depleted cells, confirming the specific involvement of OSBP in SM regulation (Figure 7A). However, OSBP with deletions or mutations of the PH, oxysterol binding, or FFAT domains did not restore 25OH stimulation of SM synthesis, even though expression levels were similar (Figure 7B). Consistent with restoration of SM synthesis, resOSBP promoted localization of CERT to the Golgi apparatus in the presence of 25OH (Figure 7C). resOSBP FF/AA was extensively localized with CERT at the Golgi in the absence and presence of 25OH, whereas resOSBP ΔPH was cytosolic under both conditions and did not promote the localization of CERT to the Golgi. Finally, resOSBP Δ432-435 was observed in punctate structures that colocalized with VAP (Figure 7D) and did not translocate to the Golgi apparatus in the presence of 25OH (Figure 7C). CERT did no localize to the Golgi apparatus in OSBP-depleted cells expressing resOSBP Δ432-435 and treated with 25OH. Thus, mutations in the dual-organelle recognition motifs of OSBP prevented localization required for activation of CERT and SM synthesis.

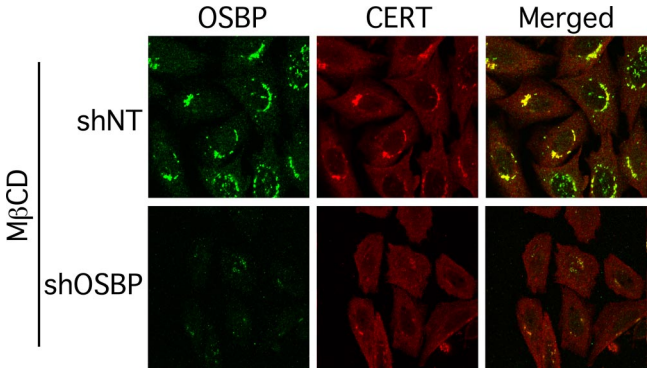


**Figure 6.** CERT translocation to the Golgi apparatus after cholesterol depletion is OSBP dependent. CHO-K1 cells stably expressing NT or an OSBP-specific shRNA (shOSBP) were treated with 5 mM M$\beta$CD for 30 min and processed for indirect immunofluorescence localization of CERT and OSBP by confocal microscopy as described in the legend to Figure 5A.

Because the OSBP FFAT domain was required for OSBP function (Figure 7A), we tested whether VAP was involved in basal or 25OH-stimulated SM synthesis using RNAi. VAP (which included A and B isoforms in CHO-K1 cells and other cell lines; Nishimura *et al.*, 1999; Wyles *et al.*, 2002) was knocked down to a similar extent (70–80%) in shNT cells, shOSBP cells, or in combination with CERT depletion by siRNA (Figure 8A). Similar to OSBP depletion by shRNA, depletion of VAP with a siRNA resulted in loss of activation of SM synthesis in response to 25OH but did not affect basal synthesis (Figure 8B). Depletion of VAP in combination with CERT or OSBP enhanced the inhibition of SM synthesis in the absence or presence of 25OH, respectively.

### *Protein Interactions between OSBP, CERT, and VAP*

Colocalization of OSBP and CERT at the Golgi apparatus in 25OH- and M$\beta$CD-treated cells, and the requirement for VAP in this process, indicated that enhanced ceramide delivery could involve interactions between these proteins. To determine whether OSBP, CERT, and VAP-A physically interact, cDNAs were fused to the GAL4 DNA (pAS1) or activation domains (pACT2), transformed into yeast, and grown on plasmid-selective media (control) or interaction-selective media (selection) (Figure 9A). Yeast expressing

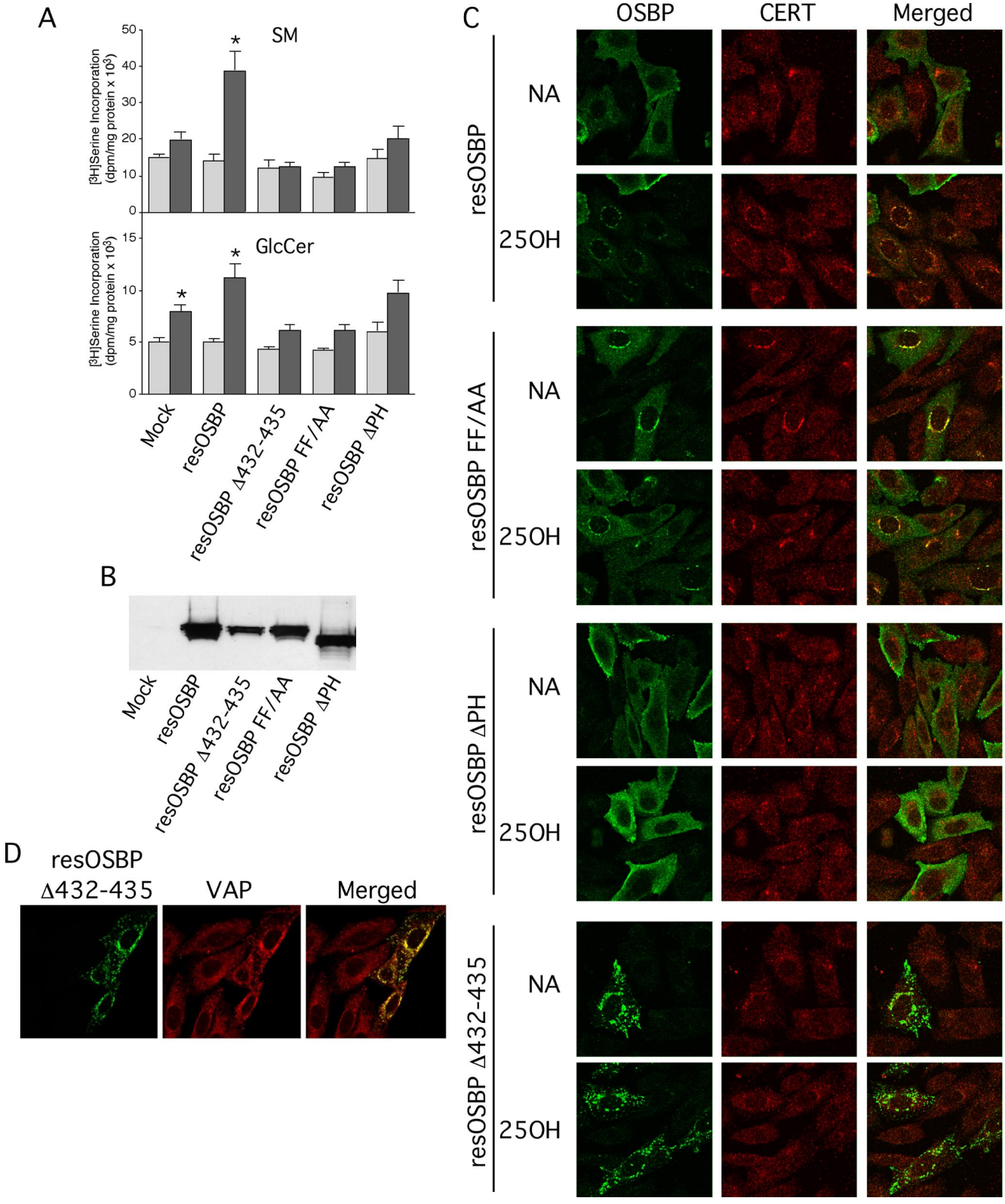


**Figure 7.** The PH, FFAT, and 25-OH binding domains of OSBP are required to reconstitute 25OH-dependent SM synthesis in shOSBP cells. (A) OSBP-depleted shOSBP cells were transiently transfected with shRNA-resistant (res) cDNAs encoding resOSBP, resOSBP Δ432-435, resOSBP FF$^{361,362}$AA (FF/AA), resOSBP ΔPH, or empty vector (mock). After 48 h, cells were incubated with 6 $\mu$M 25OH (dark gray bars) or solvent control (light gray bars), and SM and GlcCer synthesis was quantified by [$^3$H]serine incorporation as described in the legend to Figure 2. Results are mean and SE of three to six independent experiments. *$p \leq 0.001$ compared with paired solvent treated control. (B) Immunoblot analysis of wild-type and mutant resOSBP expression in shOSBP cells. (C) Transiently transfected shOSBP cells were treated with 6 $\mu$M 25OH or NA for 2 h, immunostained for OSBP and CERT, and images acquired by confocal microscopy as described in the legend to Figure 5A. (D) Untreated resOSBP Δ421-425–transfected cells were immunostained for OSBP and VAP.

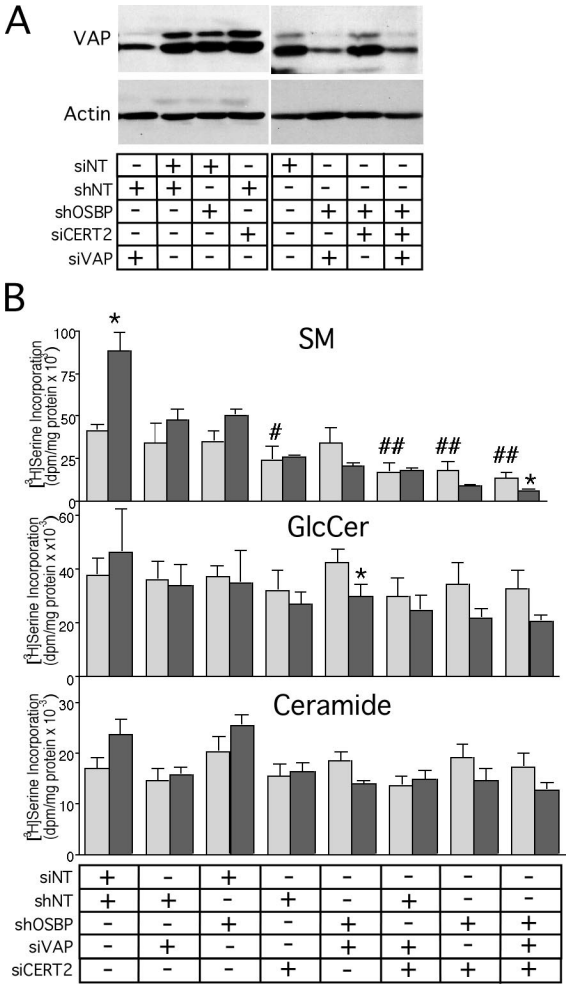


**Figure 8.** Suppression of VAP expression by RNAi blocks the 25OH-mediated increase in SM synthesis. shNT or shOSBP cells were transfected with siRNAs directed against CERT and/or VAP as described in *Materials and Methods*. (A) The expression of VAP in shRNA/siRNA-transfected cells was assessed by immunoblotting with a polyclonal antibody. The synthesis of SM, GlcCer, and ceramide was quantified by [$^3$H]serine incorporation in transfected cells treated with 6 $\mu$M 25OH (dark gray bars) or solvent control (light gray bars) as described in the legend to Figure 2. Results are the mean and SE of three separate experiments. *$p \leq 0.01$ compared with paired solvent-treated control. $^{\#}p \leq 0.02$ and $^{\#\#}p \leq 0.005$ compared with solvent-treated shNT cells.

combinations of OSBP-VAP-A, CERT-VAP-A and CERT-CERT grew on selective media indicating interaction between these proteins. No growth was observed with CERT-OSBP (lack of interaction was also observed for the pACT2-OSBP + pAS1-CERT combination) or when CERT was expressed with the empty partner vector. Immunoblotting confirmed that OSBP-, VAP- and CERT-GAL4 fusion proteins were expressed in yeast (Wyles *et al*., 2002; our unpublished data).

To confirm that CERT interacted with VAP-A via the FFAT motif, extracts of COS cells expressing wild-type CERT (CERT-V5) or the FFAT domain mutant FF$^{321,\ 322}$AA (CERT FF/AA-V5) were tested for interaction with GST-VAP-A (Figure 9B). OSBP and CERT bound to GST-VAP-A but not the GST control (Figure 9B). CERT-FF/AA-V5 was expressed to similar levels as the wild-type protein but did not bind GST-VAP-A. Next, we examined how the FFAT motif affected the cellular localization of CERT-GFP (Figure 9, C and D). CERT-GFP expressed at low levels was distributed throughout the cell and at the Golgi apparatus in a pattern similar to the endogenous protein (Figure 5). However, high expression resulted in reorganization of endogenous VAP into ER inclusions enriched in CERT-GFP (Figure 9C). These structures were either devoid of giantin or corresponded to normal Golgi containing giantin (Figure 9D). Similar VAP-enriched ER structures were observed after overexpression of OSBP W174A or Nir2 and were attributed to ER membrane stacking due to cross-bridging between VAP and these binding partners (Wyles *et al*., 2002; Amarilio *et al*., 2005). Consistent with the in vitro requirement for the CERT FFAT motif in VAP binding, CERT-FF/AA-GFP expressed in CHO-K1 cells localized exclusively with giantin at the perinuclear Golgi apparatus and did not colocalize with or affect endogenous VAP localization (Figure 9, C and D).

Finally, interaction between endogenous OSBP, CERT, and VAP was examined by in vivo cross-linking and immunoprecipitation in control, 25OH-, and M$\beta$CD-treated CHO-K1 cells (Figure 10). A VAP antibody reproducibly coimmunoprecipitated a portion of endogenous CERT from cross-linked extracts of shNT CHO-K1 cells that received no addition or carrier solvents. Prior treatment of shNT cells with 25OH or M$\beta$CD increased the amount of CERT that coimmunoprecipitated with VAP compared with solvent-matched controls by 1.8-fold (Figure 10, A and B). In contrast, CERT was immunoprecipitated weakly and variably by an OSBP antibody, and this was not affected by 25OH or M$\beta$CD (Figure 10A). When similar experiments were preformed in CHO-K1 cells expressing shOSBP, the 25OH- and M$\beta$CD-mediated increase in CERT association with VAP was prevented (Figure 10B). Thus, OSBP seems to be necessary for the formation of a CERT-VAP complex, but it displays only a weak or indirect interaction with CERT.

## DISCUSSION

Many biological activities of sphingolipids can be attributed to a strong physical interaction with cholesterol and a propensity to associate in liquid-ordered membrane microdomains, also known as rafts (Brown and London, 2000). The requirement for stoichiometric lipid and sterol composition in microdomains suggests that metabolism of cholesterol and sphingolipids is coordinately controlled. In this report, we identify one such integrative pathway that coordinates oxysterol and cholesterol signals via OSBP with CERT-dependent ceramide transport and SM synthesis at the Golgi apparatus.

The correlation between OSBP affinity for oxysterols and suppression of cholesterol synthesis indicated a role in oxysterol suppression of cholesterol synthesis (Taylor and Kandutsch, 1985). However, overexpression (Lagace *et al*., 1997) or depletion by RNAi (Nishimura *et al*., 2005) (Supplemental Figure 3) failed to demonstrate a direct role for OSBP in regulation of cholesterol synthesis by 25OH. Consequently, it is now clear that increased basal cholesterol synthesis in OSBP overexpressing cells was due to sequestration of endogenous regulatory sterols or indirectly related to increased SM synthesis. Instead of directly regulating cholesterol metabolism, OSBP is involved in sensing and integrating changes in cellular sterol levels with synthesis of SM at the Golgi apparatus by regulation of CERT-dependent ceramide transport. The finding that ceramide and SM synthesis, as well as $C_5$-DMB-ceramide fluorescence, was reduced in 25OH-

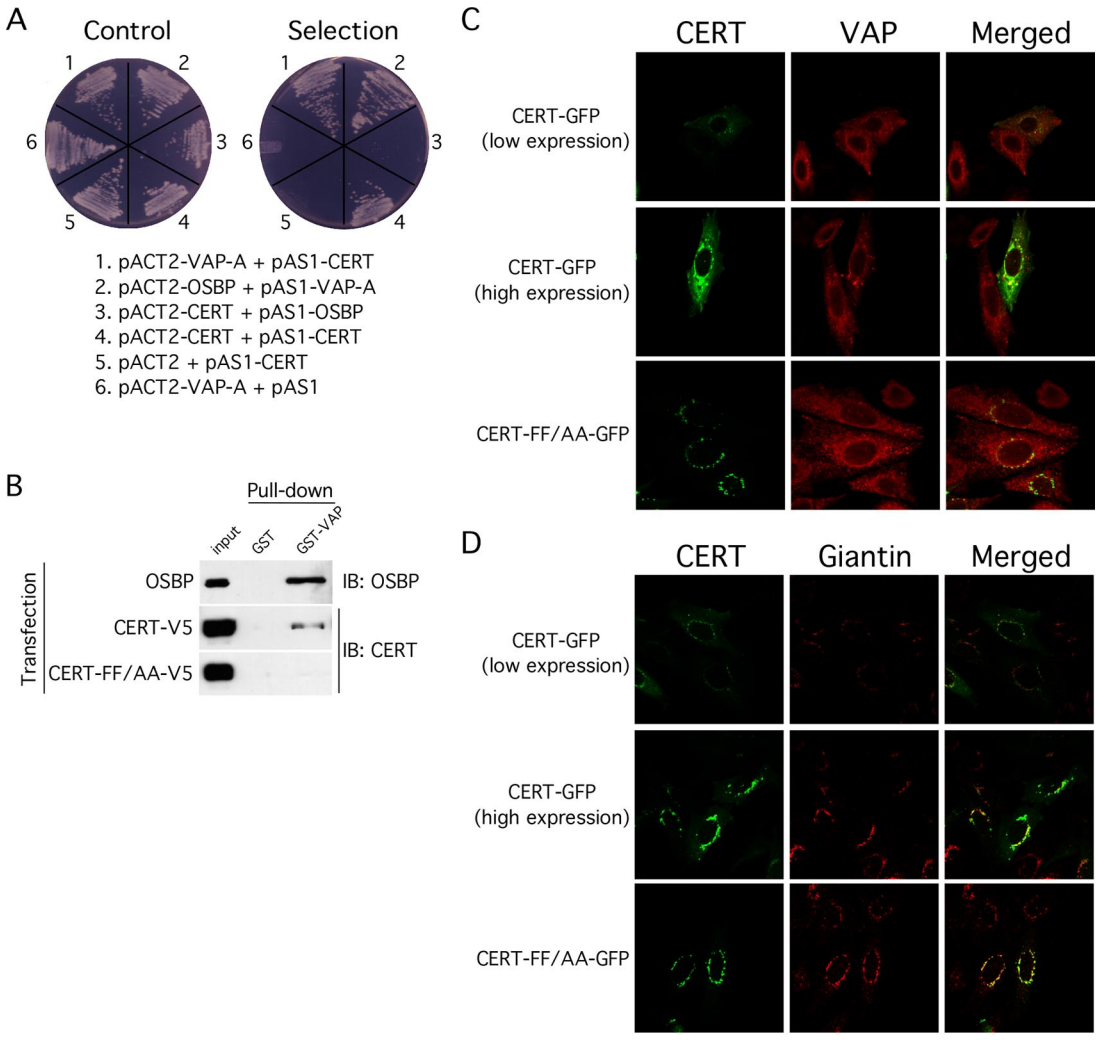


**Figure 9.** Identification of CERT, OSBP, and VAP interactions. (A) Yeast strain PJ69 transformed with two-hybrid vectors encoding OSBP, VAP, or CERT (in the indicated combinations) was sectored onto plasmid-selective media (control) and interaction-selective media (selection). A representative experiment is shown that was repeated six to eight times with similar results. (B) Interaction between CERT and VAP by GST-VAP pull-down assays. Triton X-100 extracts of COS-7 cells expressing OSBP, CERT-V5, or CERT-FF/AA-V5 were incubated with 1 $\mu$M GST-VAP-A or GST, and binding was assessed after pull-down with glutathione-Sepharose. Input represents 30% of the lysate used in the pull-down assay. (C and D) CHO-K1 cells expressing CERT-GFP or CERT-FF/AA-GFP were immunostained with VAP (C) or giantin polyclonal antibodies (D) followed by an Alexa Fluor 594-conjugated goat anti-rabbit secondary antibody. Confocal images were acquired as described in the legend to Figure 5.

treated OSBP- or OSBP/CERT-depleted CHO-K1 cells (Figures 2 and 3 and Supplemental Figure 2) also suggests that OSBP and oxysterols are involved in ceramide metabolism in the ER.

The observation that cholesterol depletion by M$\beta$CD activated SM synthesis and ceramide transport, promoted OSBP and CERT localization to the Golgi apparatus, and enhanced CERT–VAP interaction argues that this pathway also senses changes in cholesterol that are independent of oxysterols. Cholesterol depletion would not increase oxysterol levels except by derepression of de novo cholesterol synthesis. This is not the case because activation of SM synthesis by M$\beta$CD was not dependent on cholesterol synthesis (Leppimaki *et al.*, 1998). Instead, the demonstrated binding of cholesterol and oxysterols to OSBP (Wang *et al.*, 2005) and Osh4/Kes1p (Im *et al.*, 2005) suggests that OSBP and OSBP-related proteins are sensors or transporters for a number of different sterols. With this in mind, the apparent contradiction between activation of CERT and SM synthesis in response to both 25OH and cholesterol depletion by M$\beta$CD can be explained in terms of differential sterol binding. In the former case, increased SM synthesis would balance the excess cellular cholesterol signaled by oxysterols and sustain activities dependent on fixed rations of membrane SM and cholesterol. In the latter situation, increased cholesterol mobilization from the cell interior to the PM likely resulted in increased cholesterol binding to OSBP, translocation to the Golgi apparatus, and activation of CERT. It is notable that increased flux of LDL cholesterol from the lysosomes/late endosomes to the ER produced the opposite effect—OSBP dissociation from the Golgi apparatus (Mohammadi *et al.*, 2001).

RNAi, OSBP reconstitution, and immunoprecipitation experiments demonstrated that association of OSBP with VAP at the ER (Wyles *et al.*, 2002) and PtdIns-4-P/ARF (Levine

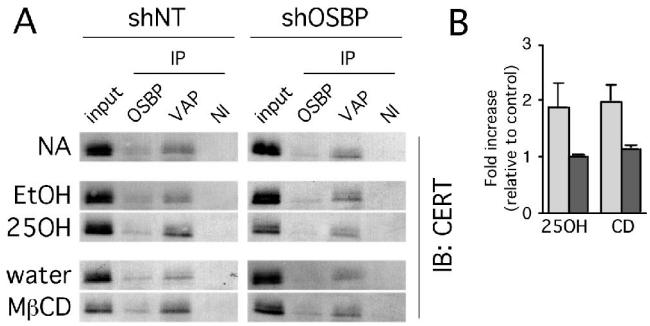


**Figure 10.** Interaction between CERT and VAP is sterol and OSBP sensitive. (A) shNT and shOSBP cells received NA or were treated with 6 $\mu$M 25OH (for 2 h), 5 mM M$\beta$CD (for 30 min), or the control solvents ethanol (EtOH) and water. Cells were incubated with 1 mM dithiobis succinimidyl propionate, extracted, and immunoprecipitated (IP) with rabbit polyclonal antibodies against OSBP, VAP, or a nonimmune rabbit serum (NI). Immune complexes were recovered on protein A-Sepharose, resolved by SDS-10% PAGE, and immunoblotted (IB) for CERT. The input lane contains 25% of the cross-linked cell extract used for immunoprecipitation. (B) Blots from three independent experiments were scanned by densitometry, and VAP association with CERT was quantified (relative to solvent-treated controls) in shNT (light gray bars) or shOSBP cells (dark gray bars).

and Munro, 2002) at the Golgi apparatus were both required for CERT recruitment to the Golgi apparatus and stimulation of SM synthesis. resOSBP FF/AA displayed Golgi localization and enhanced CERT translocation to the Golgi apparatus in the presence or absence of 25OH, yet did not restore SM synthesis. Thus, Golgi localization of CERT in the absence of OSBP–VAP interactions is insufficient to increase SM synthesis. This conclusion is reinforced by RNAi experiments showing that VAP was required for 25OH stimulation of SM synthesis (Figure 8). For CERT activity to increase there must be enhanced interaction with VAP at the ER where newly synthesized ceramide is bound. 25OH and M$\beta$CD increased the association between VAP and CERT, but not OSBP and CERT, in an OSBP-dependent manner. Recent structural studies of the FFAT motif indicate that VAP-FFAT forms a 2:2 complex that would facilitate interaction with full-length members of the OSBP family (Kaiser *et al*., 2005). Because OSBP (Ridgway *et al*., 1992; Wyles *et al*., 2002) and CERT (Raya *et al*., 2000) (Figure 8) homodimerize and interact with VAP via the FFAT motif (Figure 9), there is the potential for OSBP and CERT to form indirect interactions via heteromeric complexes with VAP. In this context, OSBP could act in a transient or catalytic manner to promote VAP multimerization and increase CERT association and ceramide loading at the ER. Although the sterol-dependent association between VAP and CERT was significant, it should be noted that only 10–20% of CERT was complexed with VAP under these conditions. Consistent with this, the majority of CERT in 25OH- or M$\beta$CD-treated cells was localized to the Golgi apparatus.

OSBP displayed weak, sterol-independent interaction with CERT; however, both translocated to the Golgi apparatus, and OSBP was clearly required for CERT recruitment to the organelle. The lack of a direct CERT–OSBP interaction, and the dependence of Golgi recruitment of CERT on the OSBP PH domain, suggest that OSBP-dependent localization of CERT to the Golgi apparatus involves increased accessibility to shared PH domain ligands. Because CERT and OSBP PH domains have similar specificity for PtdIns-4-P and would compete for binding at the Golgi apparatus, CERT recruitment is unlikely to involve the initial binding of the OSBP PH domain to the Golgi apparatus. Instead, recruitment of ARF by the PH domain of OSBP could stimulate PtdIns-4-K-III$\beta$ (Godi *et al*., 1999, 2004), causing increased localized PtdIns-4-P synthesis for recruitment of CERT. Interestingly, resOSBP FF/AA promoted the Golgi localization of CERT in the absence of exogenous oxysterol, indicating that ligand binding is not involved in CERT recruitment at the Golgi but rather for disruption of the OSBP–VAP interaction. This is supported by the constitutive localization of oxysterol binding defective resOSBP $\Delta$432-435 with VAP (Figure 7D).

Collectively, these data suggest that OSBP activation and translocation by sterols recruits an inactive pool of CERT into an active transport pathway, which under steady-state conditions is reflected by association with OSBP at the Golgi apparatus and with VAP at the ER. Current models for CERT-dependent ceramide transport involve binding and targeted diffusion across an aqueous compartment or binding to adjacent Golgi–ER membrane contact sites (Levine, 2004; Perry and Ridgway, 2005). Although perinuclear localization of VAP was observed in 25OH treated cells, it had a more diffuse staining pattern compared with the discrete Golgi localization pattern for either OSBP or CERT (Supplemental Figure 5). However, it was unclear whether the VAP that was relocalized during 25OH treatment represents enrichment at ER–Golgi contact sites that could be involved in ceramide transport. VAP and CERT displayed increased physical association in response to sterols, as would be predicted from a contact model, but the extent of association was limited to <20% of total CERT. Although this could represent a limitation of the method used to cross-link and coimmunoprecipitate VAP and CERT, it does argue against a model that involves CERT bridging between ER and Golgi membranes.

In summary, we have identified a regulatory pathway controlled by OSBP and VAP that responds to increased 25OH and cholesterol efflux by increasing CERT activity and SM synthesis. This not only defines the mode of regulation for a novel pathway involving two proteins with dual ER–Golgi targeting signals but also a mechanism for integrating cholesterol and SM metabolism.

## ACKNOWLEDGMENTS

We thank Kerry Hewitt, David Mader, and Robert Zwicker for excellent technical assistance. Steve Whitefield and Haiyun Zhang provided helpful comments on fluorescence imaging. This work was supported by an operating grant (MOP-15284) and Scientist award from the Canadian Institutes of Health Research (to N.D.R.) and a studentship from the Heart and Stroke Foundation of Canada (to R.J.P.).

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