Vesicle-associated Membrane Protein-associated Protein-A (VAP-A) Interacts with the Oxysterol-binding Protein to Modify Export from the Endoplasmic Reticulum*

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Oxysterol-binding protein (OSBP) is 1 of 12 related proteins implicated in the regulation of vesicle transport and sterol homeostasis. A yeast two-hybrid screen using full-length OSBP as bait was undertaken to identify partner proteins that would provide clues to the function of OSBP. This resulted in the cloning of vesicle-associated membrane protein-associated protein-A (VAP-A), a syntaxin-like protein implicated in endoplasmic reticulum (ER)/Golgi vesicle transport, and phospholipid regulation in mammalian cells and yeast, respectively. By using a combination of yeast two-hybrid, glutathione S-transferase pull-down and immunoprecipitation experiments, the VAP-A-binding region in OSBP was localized to amino acids 351–442. This region did not include the pleckstrin homology (PH) domain but overlapped with the N terminus of the oxysterol binding and OSBP homology domains. C- and N-terminal truncations or deletions of VAP prevented interaction with OSBP but did not affect VAP multimerization. Although the OSBP PH domain was not necessary for VAP-A binding in vitro, interaction with VAP-A was enhanced in cells by mutation of the conserved PH domain tryptophan (OSBP W174A) or deletion of the C-terminal half of the PH domain (OSBP Δ132–182). OSBP W174A retained oxysterol binding activity, association with phospholipid vesicles via the PH domain, and localized with VAP in unusual ER-associated structures. At 40 °C, misfolded ts045-vesicular stomatitis virus G protein fused to green fluorescent protein was co-localized with VAP/A/OSBP W174A structures on the ER but was exported to the Golgi when folded normally at 32 °C. A fluorescent ceramide analogue also accumulated in these ER inclusions, and export to the Golgi was partially inhibited as indicated by decreased Golgi staining and a 30% reduction in sphingomyelin synthesis. These studies show that OSBP binding to the ER and Golgi apparatus is regulated by its PH domain and VAP interactions, and the complex is involved at a stage of protein and ceramide transport from the ER.

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The abbreviations used are: OSBP, oxysterol-binding protein; C5-DMB-ceramide, N-((4,4-difluoro-5-(2-thienyl)-4-boro-3a,4a-diaza-5-indene-3-yl)phenoxo)acetyl)sphingosine; ER, endoplasmic reticulum; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; GFP, green fluorescent protein; ORP, OSBP-related proteins; OH, OSBP homology domain; PH, pleckstrin homology; Ptdlns-4-P, phosphatidylinositol 4-phosphate; Ptdlns-4,5-P2, phosphatidylinositol 4,5-bisphosphate; PtdSer, phosphatidylserine; PtdCho, phosphatidylcholine; PtdEtN, phosphatidylethanolamine; PDI, protein-disulfide isomerase; SM, sphingomyelin; VAMP, vesicle-associated membrane protein; VAP, VAMP-associated protein; VSVG, vesicular stomatitis virus G; GST, glutathione S-transferase; PBS, phosphate-buffered saline; CHO, Chinese hamster ovary; Tet, tetracycline; Dox, doxycycline; SNARE, soluble N-ethylmaleimide-sensitive factor attachment protein receptors.

Oxysterol-binding protein (OSBP)1 and 11 other related proteins constitute the recently identified OSBP-related (ORP) gene family. Family members share amino acid sequence identity in a 350-amino acid C-terminal OSBP homology (OH) domain that is encompassed in the oxysterol-binding region of OSBP. Most ORPs also contain an N-terminal pleckstrin homology (PH) domain (1–5). Initial analysis of the distribution of this gene family suggested that ORPs and OSBP share related functions in cells or have tissue-specific activities. The precise nature of these activities is unclear, but preliminary evidence in yeast and mammalian cells suggests a role in vesicular trafficking and lipid and sterol regulation (3–7).

OSBP, the first member of the gene family to be characterized, was identified by Kandutsch and Shown (8) as a high affinity cytosolic receptor for a variety of oxysterol regulators of cholesterol synthesis, such as 25-hydroxycholesterol. Based on a correlation between the potency of oxysterols to suppress 3-hydroxy-3-methylglutaryl-CoA reductase activity and affinity for OSBP, it was proposed that OSBP played a role in mediating the effects of oxysterols on cholesterol homeostasis (9). Cloning and expression studies revealed that OSBP was a soluble protein that underwent translocation from a cytosolic/vesicular compartment to the Golgi apparatus when cells were challenged with exogenous 25-hydroxycholesterol (10). Interaction with the Golgi apparatus has since been shown to involve the OSBP PH domain and is mediated by phosphatidylinositol 4,5-bisphosphate (PtdIns-4,5-P2) and possibly other unidentified protein or lipid factors (6, 11). The interaction of OSBP with the Golgi apparatus via the PH domain is important for function since overexpression of OSBP mutants with deletions of the PH domain did not cause alterations in cholesterol homeostasis that were evident with wild-type protein (6). In a model for oxysterol-mediated translocation, 25-hydroxycholesterol binding to the C-terminal region of OSBP could unmask the PH domain and thus facilitate binding to lipid or protein targets in the Golgi apparatus. Expression of the OSBP PH domain alone...
was sufficient to disrupt Golgi structure and interfered with transport of vesicular stomatitis virus G (VSV-G) protein in the Golgi apparatus (11). This suggests that an unidentified important step(s) in vesicle transport is regulated by OSBP, and this activity either directly or indirectly affects sterol regulation.

OSBP also localized to the Golgi in response to factors other than oxysterols. For instance, depletion of cellular cholesterol with cyclodextrins (12), by somatic mutations in CHO cells (13), or the Niemann-Pick C disorder (14) promoted OSBP localization to the Golgi. Because some of these models produced severe cholesterol depletion, it was unlikely that endogenous oxysterol production could account for translocation. Depletion of plasma membrane sphingomyelin (SM) and phosphatidylcholine with bacterial phospholipases also promoted translocation of OSBP to the Golgi apparatus (12). This raised the intriguing possibility that OSBP movement is linked to the production of the bioactive lipids ceramide and diglyceride. In yeast, a functional link has been established between diglyceride, phosphatidic acid, and Golgi vesicle biogenesis, mediated by the phosphatidylinositol/phosphatidylcholine transfer protein Sec14p (15–17). Deletion of the yeast OSBP homologue 4 (OSH4) bypassed the requirement for SEC14 in regulation of Golgi vesicular transport, suggesting Osh1p/Kes1p regulates Golgi secretory function in response to these lipids (18). Although none of the seven yeast OSH-encoded proteins were essential, deletion of the entire gene family was lethal (7). Depletion of all or some combinations of OSH genes was accompanied by perturbations in ergosterol metabolism and membrane function (7, 18, 19).

Although available evidence points to an important role for OSBP and related family members in lipid and cholesterol homeostasis in the Golgi/vesicular pathway, precise targets and mechanism of action are unresolved. To address this question we undertook the identification of proteins that interact with OSBP using the yeast two-hybrid method. The sole OSBP-binding protein identified in our screen was vesicle-associated membrane protein (VAMP)-associated protein-A (VAP-A), one of a pair (VAP-A and -B) of related integral membrane proteins localized to the endoplasmic reticulum (ER)/Golgi pathway (20, 21). Interaction of VAP proteins with VAMP and other components of the vesicle trafficking machinery (22) and similar topology and coiled-coil structure to the t-SNARE syntaxin family (23) suggested a role in vesicle transport. In support of this, VAP proteins have been shown to have a number of potential functions related to vesicle transport including regulation of COPI vesicle transport in the ER/Golgi pathway (21), VAMP/synaptobrevin-mediated neurotransmitter release (20), VAMP-2-mediated Glut-4 trafficking at the plasma membrane (24), and interaction with the microtubule network (25) and tight junctions (26). In the present study we define the regions of VAP-A and OSBP that mediate interaction in the Golgi apparatus, and we show that the VAP-A/OSBP interaction in vivo is regulated by the PH domain and involved at a stage of vivo apparatus, and we show that the VAP-A/OSBP interaction

VAP-A and OSBP Interaction and ER Export

VAP-A expression by immunoblotting with an anti-VAP polyclonal antibody to p58 (ER-Golgi intermediate compartment-p58) was provided by Dr. Jaakko Saraste (University of Bergen, Norway). A rabbit polyclonal to β-COP was purchased from Affinity Bioreagents Inc. N-44,4- Diufuro-5-(2-thienyl)-4-bors-3r,4o-diaza-5-indene-3'-yloxybenzoylacetate) sphingosine (6-DMB-sphingosine) was from American Plasmid Construct—pAS1-CYH2-OSBP and pACT2-OSBP were prepared by PCR amplification of the full-length rabbit OSBP cDNA using Vent polymerase (Invitrogen) and primers containing NdeI and BamHI sites (pAS1-CYH2) or NcoI and BamHI sites (pACT2) at the 5' and 3' ends of the cDNA, respectively. PCR products were subcloned into pCR2.1-TOPO prior to digestion and ligation into pAS1-CYH2 or pACT2. In order to remove the 5'-untranslated sequence from pAS1-CYH2 cloned during the two-hybrid screen, the NcoI fragment of human VAP-A cDNA was subcloned into pACT2. pAS1-CYH2-VAP was constructed by subcloning an NdeI-BamHI fragment from pACT2-VAP into pAS1-CYH2. pGEX-VAP and pTRE-VAP were prepared by PCR amplification of the VAP cDNA using Vent polymerase and primers containing EcoRI and BamHI restriction sites and ligation into the aforementioned vectors. pTRE-OSBP was prepared by ligation of an EcoRI/SspI fragment from pOSBP (10) into EcoRl/XhoI-digested pTRE (the XhoI site was made blunt by filling in with Klenow) OSBP deletion and truncations mutants in pOSBP, pTRE-OSBP, or in yeast two-hybrid vectors were prepared using the Gene Editor mutagenesis system (Promega) and confirmed by sequencing.

Two-hybrid Screen—Yeast two-hybrid vectors were transformed into yeast strain PJ69 (ATCC 21450), which harbors ADE2 and HIS3 under the control of the GAL4 promoter and will grow in the absence of histidine and adenine only in the case of positive interaction between bait and target proteins (29). Transformed yeast were grown on synthetic medium containing yeast nitrogen base (without amino acids), glucose, histidine, methionine, uracil, adenine, and lysine to confirm transformation, and on selective media containing yeast nitrogen base (without amino acids), glucose, methionine, lysine, and uracil to detect interacting partner proteins. A B-cell library in pACT (provided by Dr. Chris Barnes, Department of Microbiology and Immunology, Dalhousie University) was transformed into strain PJ69 harboring pAS1-CYH2-OSBP. Expression of the GAL4-OSBP fusion in yeast was confirmed by Western blotting using monoclonal antibody 1H9. Plasmids from yeast colonies growing on selective plates were isolated and transformed into JF1754, a DHis strain deficient in leucine biosynthesis, and grown on leucine-deficient agar plates. Plasmids isolated from JF1754 colonies were transformed into PJ69/þ/SE1112 to eliminate false positives.

Cell Culture and Transfections—CHO-Tet-On or Tet-Off cells were cultured in Dulbecco’s modified Eagle’s medium containing 5% Tet system fetal calf serum (FCS), and 33 µg/ml proline (medium A). CHO-Tet-On cells were transfected with 10 µg of pTRE-VAP and 1 µg of pTRE-Hyg using the calcium phosphate method. Cells expressing VAP were selected in medium A containing 500 µg of pTRE-VAP and 1 µg of pTRE-Hyg using Vent polymerase (Invitrogen) and primers containing NdeI and BamHI restriction sites and ligation into the aforementioned vectors. pTRE-OSBP was prepared by ligation of an EcoRI/SspI fragment from pOSBP (10) into EcoRl/XhoI-digested pTRE (the XhoI site was made blunt by filling in with Klenow) OSBP deletion and truncations mutants in pOSBP, pTRE-OSBP, or in yeast two-hybrid vectors were prepared using the Gene Editor mutagenesis system (Promega) and confirmed by sequencing.

Materials—G418, hygromycin B, tissue culture media, and secondary rabbit and mouse antibodies coupled to horseradish peroxidase were obtained from Invitrogen. Tet-On and Tet-Off system plasmids, CHO cells, and Tet system fetal calf serum (FCS) were from CLONTECH. Nutrient supplements, amino acids, and media for growing yeast were from EM Science. Protein A-Sepharose and glutathion-Sepharose 4B were from Amersham Biosciences. Protease inhibitor mixture was from Roche Molecular Biochemicals. A monoclonal antibody to protein-disulfide isomerase (PDI) was from StressGen. A vector expressing VSVG-GFP was provided by Dr. Jennifer Lippincott-Schwartz (National Institutes of Health, Bethesda) (27). A polyclonal antibody to human sec11 (28) was supplied by Dr. Fred Gorlick (Yale University, New Haven, CT). A polyclonal antibody to human OSBP was raised against a GST fusion protein containing the C-terminal 100 amino acids of human ORP1 and a pan-OSBP antibody that recognizes several OSBP family members.2 Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. Filters were incubated with primary antibodies for 1 h

2 J. P. Wyles and N. D. Ridgway, unpublished results. Antigens and Immunoblotting—GST-VAP was expressed in bacteria by isopropyl-1-thio-D-galactopronosamine induction at 25 °C for 3 h, extracted by lysozyme and Triton X-100 treatment, and purified by affinity chromatography on a glutathione-Sepharose 4B column (31). An anti-VAP antibody was prepared by immunizing rabbits with the GST-VAP fusion protein. OSBP monoclonal 1H9 was described previously (32). Antibodies and Immunoblotting—GST-VAP was expressed in bacteria by isopropyl-1-thio-D-galactopronosamine induction at 25 °C for 3 h, extracted by lysozyme and Triton X-100 treatment, and purified by affinity chromatography on a glutathione-Sepharose 4B column (31). An anti-VAP antibody was prepared by immunizing rabbits with the GST-VAP fusion protein. OSBP monoclonal 1H9 was described previously (32).
at room temperature in Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% Tween 20, and 5% (v/v) skim milk powder (Blotto). The nicolette filters were washed three times for 10 min each in Blotto, incubated for 1 h with a secondary antibody coupled to horseradish peroxidase, and washed extensively in Blotto and TBS prior to developing by the enhanced chemiluminescence method (Amersham Biosciences).

Immunoprecipitation—CHO-OSBP cells or transiently transfected COS cells were rinsed once with 2 ml of cold PBS (10 mM phosphate (pH 7.4), 150 mM NaCl) and scraped into 1 ml of PBS. The cells were collected by centrifugation for 15 min in 20 μl of lysis buffer (10 mM phosphate (pH 7.4), 150 mM NaCl, 5 mM KCl, 2 mM EDTA, 2 mM EGTA, 0.5% Triton X-100, and 1× protease inhibitor mixture). The cell lysate was subjected to centrifugation in a microcentrifuge at 14,000 rpm for 15 min at 4 °C. Supernant (20–50 μl) was incubated with 2 μl of VAP-A immune, VAP-A preimmune serum or 11H9 overnight at 4 °C, or with T7 monoclonal for 2 h at 4 °C. Protein A-Sepharose (50 μl in PBS with 0.1% Triton X-100) was added for 45 min at 20 °C. Sepharose beads were collected by centrifugation and washed three times with 1 ml of PBS containing 0.1% Triton X-100. The samples were heated to 90 °C in SDS sample buffer under reducing conditions, separated by SDS-12% PAGE and analyzed by immunoblotting.

Oxysterol and Vesicle Binding Assays—25-[3H]Hydroxycholesterol binding to OSBP in cytosol from transiently transfected COS cells was measured as described previously (6). A GST-OSBP PH domain fusion protein (GST-PH) encompassing amino acids 80–217, as well as a GST-PH W174A, was assayed for binding to phospholipid vesicles using a sedimentation assay (33). Briefly, PtdCho vesicles containing 5 mol % of other phospholipids were prepared by both sonicating and freeze-thaw cycles and incubated with purified fusion proteins (2.5 μg) in 25 mM HEPES (pH 7.4), 100 mM NaCl at 20 °C for 5 min. Samples were subjected to centrifugation at 400,000 × g for 15 min, the supernatant (unbound) removed, and the pellet (bound) resuspended by sonication in a equivalent volume of buffer. Equal volumes of both fractions were subjected to centrifugation in a microcentrifuge at 14,000 rpm for 15 min at 4 °C. Supernatant (20–50 μl) was incubated with 2 μl of VAP-A immune, VAP-A preimmune serum or 11H9 overnight at 4 °C, or with T7 monoclonal for 2 h at 4 °C. Protein A-Sepharose (50 μl in PBS with 0.1% Triton X-100) was added for 45 min at 20 °C. Sepharose beads were collected by centrifugation and washed three times with 1 ml of PBS containing 0.1% Triton X-100. The samples were heated to 90 °C in SDS-PAGE sample buffer under reducing conditions, separated by SDS-12% PAGE and analyzed by immunoblotting.

**RESULTS**

**OSBP and VAP-A Interaction**—A yeast two-hybrid screen of a human B-cell cDNA library using a GAL4 DNA binding domain-OSBP fusion as bait resulted in the cloning of 18 VAP-A cDNAs, all predicted to encode the full-length protein. VAP-A, also referred to as VAP-33 (20, 25), shares 60% identity at the amino acid level with a VAP-B or ER/Golgi 30-kDa protein (ERG30) (21). By several criteria, such as lack of OSBP interaction with irrelevant proteins fused to the GAL4 DNA binding or activation domains and the GAL4 DNA binding domain alone, and positive interaction of VAP-A with OSBP fused to either the GAL4 DNA or activation domain, VAP-A was deemed a valid candidate for further evaluation. We initially verified the interaction in vitro by GST-VAP binding to wild-type and mutant forms of OSBP in extracts from transiently transfected COS cells (Fig. 1). OSBP in the Triton X-100-soluble fraction from transfected COS cells was quantitatively recovered after binding to increasing amounts of GST-VAP but not GST or glutathione beads (Fig. 1A). OSBP with
complete or partial deletions of the PH domain or mutation of the conserved PH domain tryptophan 174 to alanine bound GST-VAP similar to the wild-type protein (Fig. 1B). OSBP truncated at amino acid 443 also bound GST-VAP, but deletions further toward the N terminus abolished binding (OSBP-1–267 and 1–217) (Fig. 1C). The quantitative nature of the in vitro interaction is demonstrated by the input into each assay shown in Fig. 1D. Collectively, these results indicate that the VAP interaction region in OSBP is between amino acids 267 and 443 and does not directly involve the PH domain.

The yeast-two-hybrid interaction assay was used to identify further the region of OSBP involved in VAP-A binding. A number of OSBP mutants fused to the GAL4 DNA binding domain (pAS1) were monitored under interaction of selective growth conditions in yeast harboring pACT-VAP or pACT2-OSBP (Fig. 2A). Based on results in Fig. 1, amino acids 261–296, which are involved in OSBP homodimerization (9), could also be involved in VAP-A binding. The OSBP-(1–261–296) construct, and two others with deletions of the N- and C-terminal halves of the dimerization domain (Δ262–278 and Δ279–296), failed to interact with wild-type OSBP but were positive for interaction with VAP-A. A deletion from 288 to 296 interacted with both VAP and OSBP, thus narrowing the OSBP homodimerization domain to amino acids 261–288. Deletion of OSBP amino acids 306–315 removed a potential SNARE V2 homology domain but did not affect interaction with either VAP or OSBP. An OSBP construct expressing amino acids 351–809 did not interact with OSBP but retained VAP interaction. In
Conjunction with GST pull-down experiments (Fig. 1), this indicated that VAP-A binding requires amino acids 351–442. This region overlaps with the ligand binding domain as well as the OSBP homology (OH) domain common to ORP family members from different species (1, 7).

Results in Fig. 2B confirmed that VAP will multimerize in a yeast two-hybrid assay (21). Truncation of VAP to remove the coiled-coil domain and transmembrane region (VAP-(1–160)), a portion of the highly conserved N-terminal sequence (VAP-(A43–49)) (35) or the transmembrane region (VAP ΔTM) abolished association with OSBP but did not affect VAP multimerization.

Association of OSBP and VAP was also confirmed by co-immunoprecipitation experiments. To facilitate these studies a polyclonal antibody was generated against the full-length VAP-A protein. By immunoblotting, this antibody recognized a protein at 30 kDa, as well as minor bands at 33 and 65 kDa (Fig. 3A). The 30-kDa protein represents the VAP-A isoform since the cDNA we cloned co-migrated with this endogenous protein when expressed in CHO cells (see Fig. 4A). The 33-kDa protein could either correspond to VAP-B or a modified version of VAP-A. Detection of both proteins was competed by inclusion of the GST-VAP fusion protein. A 65-kDa protein was observed sporadically and could be an incompletely dissociated VAP dimer (24). The VAP polyclonal antibody and OSBP monoclonal 11H9 were used to co-immunoprecipitate the complex from CHO cells stably overexpressing OSBP (Fig. 3B). Compared with direct immunoprecipitation of OSBP by 11H9, the VAP antibody weakly co-immunoprecipitated overexpressed OSBP (Fig. 3B). However, the co-immunoprecipitation was specific since OSBP was not detected using the VAP-A preimmune serum. Co-immunoprecipitation of VAP by the OSBP monoclonal 11H9 was negligible. Co-immunoprecipitation experiments were also performed using extracts from COS cells transiently transfected with OSBP and T7 epitope-tagged VAP-A and VAP ΔTM (Fig. 3C). When extracts were immunoprecipitated with the T7 monoclonal antibody, the 100-kDa OSBP was detected in both lanes by immunoblotting with monoclonal antibody 11H9. The T7 monoclonal antibody was effective in binding the majority of VAP-A in the extracts (Fig. 3C, left panel), but this resulted in co-immunoprecipitation of only 10–20% of the overexpressed OSBP (see OSBP input panel). Less OSBP was co-immunoprecipitated with VAP-ΔTM due to decreased expression of this construct relative to wild-type VAP-A. Repeated attempts to co-immunoprecipitate endogenous or overexpressed VAP using monoclonal 11H9 or other OSBP-specific antibodies were not successful. However, enhanced co-immunoprecipitation of both proteins was achieved using PH domain mutants of OSBP (see Fig. 5B).

By using a CHO cell line expressing VAP under the control of the tetracycline repressor (CHO-tet-VAP), we examined the effect of VAP-A expression on OSBP localization, as well as the localization of endogenous and overexpressed VAP (Fig. 4). In these experiments, CHO-tet-VAP cells were harvested at the indicated times after doxycycline induction, and VAP and OSBP expression in the cytosolic and Triton X-100-soluble and -insoluble membrane fractions was assessed by immunoblotting. In uninduced cells, the antibody detected two endogenous VAP proteins of 30 and 33 kDa, primarily in the Triton X-100-soluble and -insoluble fraction of 100,000 × g light membrane fraction. Induction of wild-type VAP for up to a 48-h period resulted in a progressive increase in expression of the 30-kDa protein in both heavy and light membrane fractions (Fig. 4A). Expression of the endogenous 33-kDa VAP, which appeared to be the B isoform, was not affected. In uninduced and doxycycline-induced cells, the majority of OSBP was cytosolic or in the Triton-soluble fraction of light membranes. However, induction of VAP expression for 12–48 h resulted in enhanced localization of OSBP to the Triton-soluble fraction of heavy membranes. The localization of endogenous and overexpressed VAP was monitored in these cells by indirect immunofluorescence (Fig. 4B). In uninduced cells, VAP was localized in a diffuse ER-like network, in small vesicles clustered around the nucleus and in a perinuclear/Golgi region. Induction of VAP-A expression by exposure to doxycycline for 12 h resulted in a pronounced increase in expression in the reticular network where it extensively co-localized with the ER marker PDI (results not shown).

Enhancement of OSBP-VAP-A Interaction by PH Domain Mutations—To determine the functional consequences of VAP-A and OSBP interaction, we have identified mutants of OSBP with altered affinity for VAP-A. While focusing on the PH domain of OSBP, it became apparent that mutation of the conserved tryptophan (OSBP W174) or a deletion of the C-terminal 50 amino acids of this domain (OSBPΔΔ132–182) increased affinity for membranes and altered cellular localization of OSBP. To facilitate studies of these mutant proteins, the tetracycline-inducible cell lines (Tet-Off) CHO-tet-OSBP W174A, CHO-tet-OSBP Δ132–182, and wild-type CHO-tet-OSBP were established (Fig. 5). By using a monoclonal anti-
body that recognized only the overexpressed OSBPs, it was found that the time courses for induction and level of expression of wild-type and the OSBP mutants were similar in the Tet-Off CHO cell lines (Fig. 5 A). Similar to wild-type OSBP, two molecular species were detected for the PH domain mutants indicating normal phosphorylation (36). By using a polyclonal antibody to detect both endogenous and overexpressed OSBP, the level of overexpression relative to endogenous OSBP was estimated to be 6–8-fold 3 days after removal of doxycycline (results not shown). To compare the interaction of VAP with OSBP and the PH domain mutants, extracts from CHO-Tet-Off cells (cultured in the absence or presence of doxycycline) were immunoprecipitated with antibodies to OSBP or VAP and immunoblotted with the corresponding antibodies (Fig. 5B). Similar to results in Fig. 3, it was difficult to detect co-immunoprecipitation of wild-type OSBP and VAP (a faint signal was detected upon overexposure of the OSBP blot). In contrast, OSBP W174A or Δ132–182 formed stable complexes with VAP-A that were easily detected with either combination of antibody. Because of

![Fig. 4. Enforced expression of VAP increases membrane localization of OSBP. A, CHO-Tet-VAP cells were subcultured in medium A for 24 h prior to inducing VAP-A expression with doxycycline (Dox, 2 μg/ml) for 12, 24, or 48 h. Cells cultured for 48 h in the absence of doxycycline served as controls (0 h). Cells were harvested in PBS and homogenized in lysis buffer without Triton X-100 by 20 passages through a 23-gauge needle. Homogenates were subject to centrifugation at 10,000 × g for 15 min to isolate a heavy membrane fraction. The supernatant was then fractionated into cytosol and light membranes by centrifugation at 400,000 × g for 15 min. Heavy and light membrane fractions were treated on ice with lysis buffer containing 1% Triton X-100, and soluble and insoluble fractions were separated by centrifugation at 10,000 × g for 10 min. All procedures were performed at 4 °C. Individual cell fractions (20 or 50 μg) were separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted with anti-VAP or anti-OSBP antibody 170. B, CHO-Tet-VAP cells were cultured on glass coverslips for 12 h in the presence or absence of doxycycline. Cells were processed for immunofluorescence using the VAP polyclonal antibody and a FITC-labeled goat anti-rabbit secondary antibody.

![Fig. 5. Enhanced association of VAP-A with OSBP PH domain mutants. A, CHO-tet-OSBP, CHO-tet-OSBP W174A, or CHO-tet-OSBP Δ132–182 cells were cultured in medium A without (+Dox) or with (-Dox) 1 μg of doxycycline/ml for the indicated times. Equivalent amounts of Triton X-100 extracts (15 μg of protein) of cells were separated on SDS-8% PAGE and immunoblotted with monoclonal 11H9. B, Triton X-100 extracts were prepared from cells cultured in medium A with or without doxycycline (Dox) for 24 h. Extracts were immunoprecipitated (IP) with the VAP polyclonal or OSBP monoclonal antibody 11H9 and immunoblotted (IB) with the corresponding antibody as indicated under “Experimental Procedures” and the legend to Fig. 3.]}
difficulties maintaining expression in CHO-tet-OSBP Δ132–182 cell lines, we focused on characterizing the W174A mutant in subsequent experiments.

OSBP binds oxysterol ligands in a large C-terminal region (10) and phosphatidylinositide (11, 37) and protein ligands (38) via the PH domain. 25-[3H]Hydroxycholesterol binding by OSBP W174A and Δ132–182 was measured to assess the impact of these mutations on the adjacent oxysterol binding domain (Fig. 6). Wild-type OSBP was expressed in soluble and particulate fractions from transiently transfected COS cells and displayed high affinity oxysterol activity in the former fraction. OSBP W174A and Δ132–182 mutants were expressed primarily in the particulate fraction; however, the soluble fraction displayed oxysterol binding that was relatively proportional to expression and significantly above activity in mock-transfected controls.

To determine whether the increased membrane and VAP-A binding of OSBP W174A was due to altered affinity for phosphatidylinositol ligands, binding of GST-PH domain fusion proteins to lipid vesicles was assayed (Fig. 7). In these studies GST-PH or GST-PH W174A was incubated with increasing concentrations of PtdCho vesicles, supplemented with 5 mol % of the indicated phospholipids, and fusion proteins bound to vesicles were separated by centrifugation and analyzed by SDS-PAGE. Both fusion proteins bound quantitatively to vesicles supplemented with PtdIns-4,5-P_2 and PtdIns-4-P at the lowest vesicle concentration (0.25 mM). Binding to vesicles supplemented with phosphatidylserine (PtdSer) or phosphatidylinositol was relatively weak and increased slightly with vesicle concentration. PH domain binding to lipid vesicles was specific as indicated by lack of interaction of GST with PtdIns-4,5-P_2-supplemented vesicles. Results shown in Figs. 6 and 7 demon-
strate that the W174A mutation did not affect the oxysterol or phosphatidylinositol binding activity of OSBP.

**OSBP W174A Alters VAP Localization and Export from the ER**—The site of interaction between VAP-A and OSBP was determined by indirect immunofluorescence in Tet-Off cell lines (Fig. 8). In cells expressing wild-type OSBP following removal of doxycycline, there was strong co-localization of OSBP and VAP in the reticular network and a region adjacent to the nucleus. VAP-A localization in this cell line was similar to CHO-K1 cells and was not altered by induction of OSBP expression. VAP-A localization in CHO-tet-OSBP W174A cells cultured in the presence of doxycycline was similar to uninduced CHO-tet-OSBP cells (results not shown). When expression was induced by removal of doxycycline, OSBP W174A was confined to a heterogeneous population of "vesicle-like" structures, with some cells also having tubules that contained the OSBP mutant (see Fig. 9). Occasional small vesicles could be observed that lacked interior staining (see Fig. 10), indicating OSBP W174A binding to a surrounding membrane, whereas most of the large structures had OSBP localized throughout. VAP-A was strongly co-localized with OSBP W174A in all these structures confirming the enhanced interaction observed by co-immunoprecipitation (Fig. 5B).

The unusual nature of the OSBP W174A/VAP-A structures, and the association of both proteins with membranes, suggested that these represent a modified organelar membrane domain. To determine the origin of these structures, co-immunofluorescence localization of VAP or OSBP W174A with a variety of organelle markers was examined in CHO-tet-OSBP W174A cells (Fig. 9). The luminal ER redox enzyme PDI co-localized with VAP in the inclusions but only weakly elsewhere in the cell. The ER-Golgi intermediate compartment resident protein p58 (39) and β-COP, a coatomer protein found primarily in the Golgi apparatus (40), did not overlap with OSBP W174A. Sec31, a component of the COPI coat localized to ER export sites (41), was distributed in small punctate structures throughout the cell. Some of these structures co-localized with OSBP W174A. However, this was likely nonspecific and due to the high density of sec31 staining. With the exception of PDI, the localization of proteins shown in Fig. 9 was indistinguishable from uninduced CHO-tet-OSBP W174A cells or CHO-tet-OSBP cells. This suggests that the VAP-A/OSBP W174A structures are due to distortion of a region of the ER membrane caused by expression of the mutant OSBP.

**Effect of OSBP W174A on Protein and Ceramide Export from the ER**—We used ts045-VSVG tagged on the cytoplasmic tail with GFP to monitor the effect of the OSBP W174A on membrane protein export from the ER (27) (Fig. 10). At 40 °C ts045-VSVG-GFP was blocked at a late step in the folding pathway and retained in the ER in a misfolded state (42). Correct folding of VSVG and export to the Golgi apparatus and plasma membrane requires PDI, BIP, and calnexin (43). VSVG-GFP was transiently transfected into CHO-tet-OSBP or OSBP W174A cells cultured in the absence of doxycycline at 40 °C and subsequently shifted to 33 °C to promote export. In CHO cells expressing OSBP at 40 °C, VSVG-GFP was found in small vesicles and the ER. Upon shifting to 32 °C for 1 h, VSVG-GFP exited the ER and was strongly localized to the Golgi apparatus and, to a lesser extent, the plasma membrane. In CHO-tet-OSBP W174A cells at 40 °C, VSVG-GFP was localized to structures that coincided with OSBP W174A and the ER network. Refolding of VSVG-GFP at 32 °C resulted in normal export to the Golgi apparatus and plasma membrane and no residual localization to OSBP W174A structures. Thus the ER structures formed by VAP-A/OSBP W174A accumulated misfolded VSVG-GFP, but folding proceeded normally at the permissive temperature.

Ceramide is synthesized in the ER and exported to the Golgi apparatus where it is the precursor for sphingomyelin and
glycosphingolipid synthesis (reviewed in Ref. 44). OSBP and 25-hydroxycholesterol stimulated de novo SM synthesis by a process that involved increased ceramide export to the Golgi apparatus (45), and thus we were interested in the effect of the OSBP W174A mutation on ceramide transport and SM synthesis (Figs. 11 and 12). Cells were labeled with C_2-DMB-ceramide at 4 °C, and localization of the fluorescent lipid was examined immediately (0 h) or after 1 h at 37 °C (Fig. 11). In CHO-tet-OSBP cells, C_2-DMB-ceramide staining of the Golgi apparatus was increased after 1 h, and this was not affected by enforced expression of OSBP (Fig. 11, –Dox). CHO-tet-OSBP W174A cells grown in the presence of doxycycline displayed a similar increase in C_2-DMB-ceramide staining of the Golgi apparatus after 1 h. Inducible expression of OSBP W174A did not affect the initial localization of C_2-DMB-ceramide (0 h); however, after 1 h at 37 °C there was prominent staining of structures (Fig. 11, indicated by arrows) that corresponded to the ER inclusions caused by the OSBP W174A/VAP-A complex. Ceramide staining of these structures was evident after 10 min at 37 °C (results not shown). C_2-DMB-ceramide was transported to the Golgi apparatus in some cells as indicated by perinuclear staining, but this was reduced or disorganized compared with controls. Synthesis of SM was measured by [3H]serine incorporation as an index of ceramide export to the Golgi apparatus in CHO-tet-OSBP and OSBP W174A cells (Fig. 12). Consistent with what appeared to be partial suppression of fluorescent ceramide export to the Golgi apparatus and accumulation in ER inclusions, induction of OSBP W174A expression (Fig. 12, –Dox) resulted in a significant 30% reduction in de novo SM synthesis. This was not evident in cells expressing wild-type OSBP, nor was the incorporation of [3H]serine into other phospholipids affected under any conditions.

**DISCUSSION**

By virtue of its oxysterol binding activity and altered localization and phosphorylation in response to cholesterol levels and transport, OSBP has been implicated in regulation of lipid and sterol homeostasis. This could be the result of interaction with components of the vesicle transport machinery since OSBP localized to the Golgi apparatus in an oxysterol-dependent manner (10), and the OSBP PH domain was shown to alter secretory function when expressed in cells (11). In further support of this conclusion, we have shown that OSBP interacts with VAP-A and that OSBP PH domain mutations constitutively interact with VAP in the ER resulting in aberrant compartmentation and export of VSVG and ceramide.

VAP-A is a type II integral membrane protein with a large N-terminal domain facing the cytoplasm and a small 4-amino acid C terminus (21). VAP contains three distinctive domains as follows: an 18-amino acid segment proximal to the N terminus that is highly conserved among different species (35), a coiled-coil motif, and a transmembrane region at the C terminus. The function of these domains is unknown, but their deletion effectively blocked OSBP binding, but not VAP multimerization, as measured by yeast two-hybrid interaction (Fig. 2) (21). In mammalian cells, VAP has been implicated in vesicle transport but at precisely which stage is unknown (20, 21, 24). In the case of VAP-B, neutralizing antibodies as well as recombinant VAP-B were shown to inhibit in vitro intra-Golgi transport (21). The accumulation of COPI-coated vesicles in the presence of VAP antibody suggested that VAP was involved in the uncoating of vesicles during retrograde transport between the Golgi apparatus and ER. Whether VAMP or another SNARE is involved in these activities is unclear since VAMP and VAP did not co-localize in cells, and reported interactions with other components of the vesicle transport machinery were in vitro (22, 23).

Analysis of OSBP mutants by yeast two-hybrid and GST pull-down assays was used to identify a region between amino acids 351 and 442 that mediated VAP-A binding. This region overlapped with the N terminus of the oxysterol binding domain and included a portion of the OH domain between amino acids 416 and 442 that is shared by all OSBP family members (1). We have evidence that two other OSBP family members interact with VAP in yeast two-hybrid assays and by immunoprecipitation thus supporting the concept that the OH region is involved and that VAP is a partner for other OSBP family members. The truncation mutant OSBP-(1–442) did not bind oxysterols (10), but a similar truncation mutant (OSBP-(1–441)) interacted with GST-VAP, indicating that intact oxysterol binding activity is not necessary for VAP-A binding. In
vitro VAP-A/OSBP interaction did not require the PH domain; however, mutations of this domain caused enhanced localization to ER-associated structures in intact cells. A possible explanation for this result is that the PH domain is required for targeting of OSBP to the Golgi apparatus, whereas VAP-A is primarily involved in OSBP interaction at the ER. In support of this concept the isolated OSBP PH localized to the Golgi apparatus in cells and to isolated Golgi membranes in vitro (11). In CHO cells, endogenous VAP co-localized with OSBP in a diffuse reticular network but also in a perinuclear region that could correspond to the Golgi apparatus. When the OSBP PH domain was mutated, it appeared that the VAP-A interaction was dominant, and OSBP was constitutively localized with VAP-A in ER structures. This is not a simple matter of competition for OSBP binding to two receptors since complete deletion of the OSBP PH domain rendered the protein cytoplasmic (6), and the W174A mutant retained binding to PtdIns-4,5-P2, a proposed target in the Golgi. Instead, our results suggest that another unidentified activity associated with the PH domain is involved in stabilizing the interaction with VAP-A. This could be another protein or lipid partner whose affinity is altered by the W174A mutation or truncation of the PH domain.

The appearance and accumulation of misfolded VSVG and ceramide in ER inclusions formed by constitutive interaction between VAP-A and OSBP suggests a role in an ER export or protein maturation pathway. This would occur at a relatively early stage since these complexes did not involve ER export sites (sec31) or the ER-Golgi intermediate compartment (p58). The presence of misfolded VSVG suggests that OSBP/VAP-A is involved in a protein folding pathway; misfolded VSVG was present in the complexes, as were PDI and other soluble luminal chaperones (HSP 90, results not shown). As stated above, VSVG-GFP could be non-specifically concentrated in these structures and not distinguish them from the normal ER. However, a relationship between SCS2, the yeast VAP homologue, and the unfolded protein response suggests that mammalian VAP and OSBP could have a related function (49). Deletion of SCS2 resulted in inositol auxotrophy above 34 °C, a defect that was rescued by Ino1p overexpression. Genetic interactions between SCS2, phospholipid regulation, and the unfolded protein response suggests that mammalian VAP and OSBP could have a related function (49). Deletion of SCS2 resulted in inositol auxotrophy above 34 °C, a defect that was rescued by Ino1p overexpression. Genetic interactions between SCS2, phospholipid regulation, and the unfolded protein response were indicated by the rescue of inositol auxotrophy in *IRE1/HAC1* mutants by Scs2p overexpression (49). Ire1p is a transmembrane protein kinase/endoribonuclease that transduces the signals for the unfolded protein response from the ER lumen to the nucleus by assisting in splicing of the *HAC1* mRNA.
transcription factor mRNA, resulting in increased protein chaperone expression and lipid synthesis (50). Increased lipid synthesis could expand the ER to accommodate the increased lipid regulation and the unfolded protein response in yeast, and together with our data demonstrate that interaction between lipid regulation and the unfolded protein response in the ER to the Golgi apparatus. One such stress-activated response that affects the export of specific proteins from the ER is regulated by oxysterols (52), but it is unknown if VAP or OSBP are involved in this process.

Recently, a large scale characterization of yeast multiprotein complexes included analysis of a complex that was isolated using Scs2p as bait (53). This resulted in identification of nine Scs2p interacting components that included Osh1p and Osh2p, members of the yeast OSBP family that contain N-terminal PH and ankyrin domains (7). Although precise relationships between members of this complex remain to be determined, it is notable that the complex also contained Opi1p, a negative transcription regulator of inositol metabolism (54), and Stt4p, a phosphatidylinositol 4-kinase (55). These findings further confirm that OSBPs and SCs2 are involved at the interface between lipid regulation and the unfolded protein response in yeast, and together with our data demonstrate that interactions between VAP and the ORP family is conserved among yeast, and together with our data demonstrate that interaction between OSBP and VAP could mediate a similar stress-activated response that affects the export of specific proteins from the ER to the Golgi apparatus.