The N Terminus Controls Sterol Binding while the C Terminus Regulates the Scaffolding Function of OSBP

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Previously we reported that when cell cholesterol is acutely lowered with β-methyl-cyclodextrin the amount of activated ERK1/2 in caveolae dramatically increases. We traced the origin of this novel method of pERK1/2 accumulation to a macromolecular complex with dual specific phosphatase activity that contains the serine/threonine phosphatase PP2A, the tyrosine phosphatase HePTP, the oxysterol-binding protein OSBP and cholesterol. When cell cholesterol is lowered, or oxysterols is introduced, the complex disassembles and pERK1/2 increases. In an effort to better understand how OSBP functions as a cholesterol-regulated scaffolding protein, we have mapped the functional parts of the molecule. The command center of the molecule is a centrally located, 51 amino acids (408–459) long sterol-binding domain that can bind both cholesterol and 25-hydroxycholesterol. This domain is functional whether attached to the N- or the C-terminal end of OSBP. Introduction of a Y458S mutation impairs binding. Even though 25-hydroxycholesterol will compete for cholesterol binding to OSBP408–809, it will not compete for cholesterol binding in full-length OSBP. Upon further analysis we found that a glycine-alanine-rich region at the N-terminal end of OSBP works with the PH domain to control cholesterol binding without affecting 25-hydroxycholesterol binding. Finally, we found that HePTP and PP2A bind the C-terminal half of OSBP, HePTP binds a coiled-coil domain (amino acids 732–761), and PP2A binds neither the coiled-coil nor HePTP. On the basis of this information we propose a new model for how OSBP is able to sense both membrane cholesterol and oxidized sterols and link this information to the ERK1/2 signaling pathway.

Recently we proposed a model for how sterol binding to oxysterol-binding protein (OSBP)2 is linked to a critical signaling pathway in the cell (1). Conditions that lower the cholesterol level of the plasma membrane (in particular, caveolae cholesterol) cause a dramatic increase in the level of cellular pERK1/2, which is followed by a marked increase in DNA synthesis (2). The cause of the pERK1/2 increase is the loss of an enzyme activity that is able to remove phosphate from both threonine 185 and tyrosine 187 in human pERK2 (3). Instead of being a single protein, however, the dual phosphatase activity involves two separate sets of enzymes. One is the multiprotein, threonine phosphatase PP2A. The other is a member of the PTPPBS family (4) of tyrosine phosphatase. These proteins work cooperatively as part of a high molecular weight complex (~440 kDa) to dephosphorylate pERK1/2 when plasma membrane cholesterol is normal. Cholesterol depletion, however, silences the dual specific activity and, concomitant with the loss of cholesterol, the high molecular weight complex disassembles. Therefore, the cooperative behavior of the two enzymes is dependent on a cholesterol binding, scaffolding protein that holds the two phosphatases in the active configuration only when cholesterol is bound. A protein with the expected scaffolding activity was identified as OSBP (1).

A lipid-sensing protein is one where the occupation of a binding pocket by a specific lipid causes the molecule to undergo a conformational change that conveys new functional activity. A well-characterized example of this type of molecule is the sterol-sensor family of membrane proteins (5). These are polytopic membrane proteins that contain a sterol-sensing motif within the spanning regions. In the case of the sterol sensors SCAP and HMG-CoA reductase, cholesterol binding to this motif causes each to bind one of the two isoforms of the integral membrane protein Insig. Whereas the binding of Insig to HMG-CoA reductase stimulates reductase degradation, Insig binding to SCAP causes retention of the transcription factor SREBP in the ER. Importantly, these sterol sensors appear to be specialized to sense the local level of sterol in the membrane where they reside (5).

In contrast to SCAP and HMG-CoA reductase, our work (1) and others (6–8) suggests OSBP is a cytosolic sterol sensor. OSBP is a member of a protein family (referred to as OSBP-related proteins (ORPs)) that is widespread in nature with sixteen in humans, six in plants, and seven in yeast (9). OSBP is 809 amino acids long (Fig. 1) and contains an N terminus (1–88) rich in glycine and alanine (Gly-Ala domain), a PH domain (90–183), a leucine zipper (209–244), a FFAT motif (360–364) that binds the ER protein VAP, and a sterol-binding region (408–809). Originally discovered as an oxysterol-binding protein (10, 11), the crystal structure of the yeast ORP Osh4 indicates that the sterol-binding pocket of OSBP may be able to accommodate a variety of sterols including cholesterol.

8 The abbreviations used are: OSBP, oxysterol-binding protein; ERK, extracellular signal-regulated kinase; ER, endoplasmic reticulum; ORP, OSBP-related protein; HA, hemagglutinin; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)]ethyl]glycine; NTA, nitritriacetic acid; ANOVA, analysis of variance.

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2 The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S3.

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principle function of OSBP and other ORPs is thought to be to move sterols between different membrane compartments, although experiments in yeast “suggest that elimination of Osh proteins has an impact on the ability of the PM to sequester sterols but does not directly affect the transport of sterols” (12).

Here we present new evidence in support of our model that OSBP functions as a sterol sensor. We map the sterol-binding region of OSBP to an area between residue 409 and 459 and show that it is this sequence that conveys stereospecific cholesterol and oxysterol binding. The binding site for the two OSBP client enzymes (HePTP and PP2A) resides between 459 and 809. Finally, we present evidence that the alanine/glycine-rich region at the N terminus (10) controls the binding of cholesterol, but not 25-hydroxycholesterol, to the sterol-binding site in OSBP.

**EXPERIMENTAL PROCEDURES**

**Materials**—Monoclonal anti-V5 antibody was from Invitrogen (Carlsbad, CA). Monoclonal Abs for HA and Myc were from UBI (Lake Placid, NY). PP2A, C mAb was from BD Biosciences (San Jose, CA) and PP2A, B55α was a gift from Dr. Estelle Sontag at the University of Texas Southwestern Medical Center. Poly-Prep chromatography columns were from Bio-Rad and Ni-NTA beads were purchased from Qiagen (Valencia, CA). Cholesterol and 25-hydroxycholesterol were from Sigma. Epicholesterol was from Steraloids Inc. (Newport, RI). 1, 2-[3H]-(N)-cholesterol and 25- (26, 27-[3H])hydroxycholesterol were from PerkinElmer (Boston, MA). Protease inhibitor mixture set III and PMSF were from Calbiochem.

**Construction of Recombinant OSBPs**—The key OSBP cDNA constructs used in this study, which were derived from a rabbit sequence (13), are shown schematically in supplemental Fig. S3. For bacterial-expressed protein, all clones were inserted into the pET102-V5-His bacterial expression vector and expressed in Escherichia coli strain BL21 or Rosetta. pET102-OSBP-V5-His was constructed as previously described (1). pET102-OSBP-V5-His was used as the template to generate a set of subclones. To obtain pET102-OSBP1–473-V5-His, the pET102-OSBP-V5-His was digested with HindIII, partially digested with XbaI, and the 5′ overhang of the purified large XbaI-HindIII fragment was filled in and ligated. To obtain pET102-OSBP1–409-V5-His, pET102-OSBP-V5-His was digested with NcoI and the 5′ overhangs of the purified larger NcoI fragment was filled in and ligated. PCR was performed to obtain pET102-OSBP1–409-V5-His, pET102-OSBP311–409-V5-His, pET102-OSBP408–409-V5-His, pET102-OSBP298–409-V5-His, pET102-OSBP181–409-V5-His, and pET102-OSBP181–809. The clones were constructed using a QuickChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) with the following PCR primers respectively; 5′-GGAGAGATATACATAATGGCCAAGG-3′ forward, 5′-GGTTAGAAGAGAGATACATATGCAAGTCTG-3′ forward, 5′-GAGGATAATATATGGGGAGAACCAGAATCCACATGACAAG-3′ forward, and 5′-CATTAGAAGAGAGATACATATGCAAGTCTG-3′ forward.

**Scaffolding Function of OSBP**

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**Sterol Binding Assay**—Expression of cDNA inserts in pET102 is induced by isopropyl-1-thio-β-d-galactopyranoside for 3–4 h at room temperature. Bacteria were collected and resuspended in buffer A (50 mM NaH2PO4, 300 mM NaCl, pH 7.9, 10 mM imidazole, 0.2% protease inhibitor mixture set III, and 1 mM phenylmethylsulfonyl fluoride). Bacteria were disrupted by sonication and centrifuged at 4,000 × g for 25 min to remove debris. The supernatant fraction was further centrifuged at 200,000 × g for 30 min. The clear supernatant fraction was assayed for protein concentration and OSBP construct expression level before each experiment. For each binding assay, the protein concentration of the lysate was kept at 1.8 mg/ml. To achieve similar concentrations, the constructs expression level was checked by semi-quantitative immunoblotting, and the highest expressing construct in each experiment was diluted with bacterial lysates from non-expressing cells. Sterol binding was measured by placing 9.5 ml of lysate in a 15-ml conical tube, adding the indicated concentration of [3H]sterol in 20 μl of 100% ethanol, and rotating the sample for 2 h at room temperature. Subsequently, 200 μl of Ni-NTA beads (prepared according to the manufacturer’s instruction) was added to each tube, and the rotation continued for an additional 1 h at 4 °C. The sample was then centrifuged at 500 × g for 2 min to sediment the beads, and the supernatant fraction designated the unbound fraction. The beads were resuspended in buffer A and loaded onto a poly-prep chromatography column. Following three washings with 10 mM imidazole, once with 20 mM imidazole and once with 40 mM imidazole in buffer A, the bound His (6)-tagged proteins were eluted with 160 mM imidazole in buffer A. The tritium radioactivity in the elution was then measured.
measured by scintillation counting. Instead of using the columns, the beads can also be washed by low speed centrifugation (500 × g, 2 min) using the same washing procedure. Then the beads were resuspended in 160 mM imidazole and centrifuged at 500 × g for 2 min. The radioactivity in the supernatant/elution was determined by scintillation counting. Immunoblotting with a V5 mAb was performed to confirm equal amounts of His6-tagged OSBP protein in each assay of the same experiment with a V5 mAb was performed to confirm equal amounts of His6-tagged OSBP protein in each assay of the same experiment. For each experiment, the lysate from non-expressing/control cells was employed for a parallel binding assay and the radioactivity in the elution was considered as the background. OSBP-specific 3H binding was obtained by subtracting the background radioactivity from the total radioactivity associated with the OSBP samples. The OSBP-specific bound tritium was then converted into fmol of sterol. Each binding curve was plotted with GraphPad Prism software, and the binding \( K_d \) was estimated using a nonlinear regression curve. With the Bradford protein assay, we estimated that 10–20 \( \mu \)g of OSBP construct was present in each assay and that the final ethanol concentration in each experiment was \( \sim 0.5\% \). In tests not shown, we found that as much as 2% ethanol had no effect on the sterol binding characteristics of both wild type and mutant OSBPs. Depending on the sterol affinity and expression level, background binding (bacterial lysate alone) values for 25-hydroxycholesterol ranged from 1–18% and for cholesterol from 4–26%. A compilation of the binding experiments for the various constructs is shown in supplemental Fig. S3.

**OSBP-HePTP/PP2A Interaction Assay**—The HePTP/PP2A-OSBP interaction assay was carried out in HeLa cells as previously described. Briefly, cells were transfected with the indicated cDNA using Superfect transfection reagent, cultured for 24 h, washed with phosphate-buffered saline, resuspended in Buffer B (20 mM Tricine, pH 7.8, 250 mM sucrose, 1 mM EDTA, and 0.5 mM phenylmethylsulfonyl fluoride), and homogenized by nitrogen bomb at 500 psi for 15 min on ice. The homogenates were centrifuged for 30 min at 200,000 × g and the His-tagged OSBP construct isolated from the supernatant fraction using Ni–NTA beads as previously described (1).

**Other Methods**—We used a one-way ANOVA followed by multiple comparisons using Fisher’s Least–Significant–Difference Test to analyze sterol binding to both normal and mutant OSBP (supplemental Fig. S1).

**RESULTS**

**Localization of the Sterol-binding Site**—We reported previously that cholesterol promotes assembly of OSBP/HePTP/PP2A while 25-hydroxycholesterol causes disassembly (1), which implies that both sterols interact with OSBP. Recently the crystal structure of Osh4 was solved (14) and a sterol-binding pocket defined (Fig. 2). Consistent with our observation, this ORP can bind both cholesterol and oxysterol. The region of OSBP that is homologous to Osh4 (Fig. 2) encompasses amino acids 408–809 (OSBP_{408\ldots809}) and contains the previously defined oxysterol-binding region of the molecule (13, 15, 16). To see if OSBP_{408\ldots809} will also bind cholesterol, we developed a method for measuring sterol binding to bacterial expressed OSBP_{408\ldots809} (see “Experimental Procedures”). We tested the method by measuring the ability of OSBP_{408\ldots809} to bind 25-[\textsuperscript{3}H]hydroxycholesterol as a function of sterol concentration (Fig. 3A). Binding was saturable with an average half-maximal binding (apparent \( K_d \)) of 5 nm (Fig. 3C), which is comparable to the binding of 25-hydroxycholesterol to full-length OSBP expressed in COS-M6 cells (13). OSBP_{408\ldots809} also bound cholesterol (Fig. 3B). A competition binding assay was used to determine if cholesterol and 25-hydroxycholesterol bound to the same site on the molecule (Fig. 3D). Both unlabeled cholesterol and 25-hydroxycholesterol competed for [\textsuperscript{3}H]cholesterol binding to OSBP_{408\ldots809}. Epicholesterol, which is an optical isomer of cholesterol, failed to compete for binding. On the other hand, cholesterol did not compete for binding of 25-hydroxycholesterol to OSBP (E). We do not know the reason for this anomaly, but it may be due to the low solubility and low affinity of cholesterol compared with 25-hydroxycholesterol. Nevertheless, OSBP_{408\ldots809} appears to have a single, stereospecific, sterol-binding site that can bind either cholesterol or oxysterols.

The ability of OSBP_{408\ldots809} to bind both cholesterol and 25-hydroxycholesterol is in agreement with the structural properties of the sterol-binding pocket found in Osh4 (14). Osh4 predicts that the \(-\)barrel region of OSBP between amino acids 458 and 694 is the sterol-binding site (Fig. 2). A convenient restriction site allowed us to construct a cDNA coding for amino acids 1–473 of OSBP (OSBP<sub>1–473</sub>), which does not include any part of the \(-\)barrel region (Fig. 2). This construct clearly binds 25-hydroxycholesterol (Fig. 4A and supplemental Figs. S1 and S3) with an average \( K_d \) of 122 nm (Fig. 4C and supplemental Fig. S1). It also binds cholesterol (Fig. 4B) with a \( K_d \) of \( \sim 216 \) nm. If, on the other hand, the first 458 amino acids of OSBP (OSBP<sub>459–809</sub>) is deleted, sterol binding is lost (Fig. 4, B and C). We also did not detect sterol binding to amino acids 1–409 (Figs. 4C, 5B, and 6A). The lack of sterol binding to both OSBP<sub>1–409</sub> and OSBP<sub>459–809</sub> suggests there is a single sterol-binding site in OSBP between amino acid 408 and 459 that will bind sterol when attached to either the N-terminal or the C-terminal one-half of the molecule. According to the
model of Osh4 (14), OSBP$_{409-459}$ maps to the putative lid region of OSBP (Fig. 2). Based on this analysis, the β barrel region is not the primary sterol-binding site in OSBP.

**Regulation of Sterol Binding**—With the sterol-binding site in the middle of the molecule it is possible that the C- and N-terminal portions of OSBP are specialized to regulate specific functions of the molecule. Two functions of interest are HePTP/PP2A binding and sterol-binding. Fig. 5A shows that full-length OSBP binds 25-hydroxycholesterol (●). It also binds cholesterol (○, Fig. 5B). The average of multiple trials indicates that in our assay full-length OSBP has a $K_d$ of ~37 nM for 25-hydroxycholesterol and ~173 nM for cholesterol (Fig. 5C and supplemental Figs. S1 and S3). Ridgway et al. (15) reported that OSBP lacking amino acids 455–809 (OSBP$_{1-454}$), which would delete four amino acids from the C-terminal end of the sterol-binding domain we have localized to OSBP$_{409-459}$, does not bind oxysterols. We noticed that this set of amino acids contains a tyrosine residue at position 458 within a consensus CRAC motif (cholesterol recognition/interaction amino acid consensus: (L/V)X$_{1-5}$YX$_{1-5}$(R/K)). CRAC domains have been implicated in cholesterol binding to peripheral-type benzodiazepine receptor (PBR) (17) and Niemann-Pick C2
Therefore, we constructed an OSBP with an amino acid substitution at position 458 (Y458S) and another with a substitution at 298 (Y298S) within a putative CRAC domain outside of the sterol-binding motif. Each was then tested for oxysterol binding (Fig. 5D). Whereas OSBPY298S exhibited normal, saturable 25-hydroxycholesterol binding with a Kd of 15 nM (●), only nonspecific binding was detected for OSBPY458S (Œ). Similar results were obtained for [3H]cholesterol binding (supplemental Fig. S2). These results suggest a role for tyrosine 458 in sterol binding to OSBP.

Unlike OSBP408–809 (Fig. 3D), 25-hydroxycholesterol competed poorly for cholesterol binding to full-length OSBP (Fig. 5E). Therefore, we explored the possibility that the N-terminal end of OSBP influenced sterol binding to the sterol-binding site in OSBP408–459. Fig. 6A shows the 25-hydroxycholesterol binding curve for an OSBP that lacks the first 310 amino acids (OSBP311–809, ■) compared with OSBP1–409 (▲). The estimated Kd for 25-hydroxycholesterol binding to OSBP311–809 was ~20 nM, but in the same experiment this sterol did not bind OSBP1–409. OSBP311–809 also bound cholesterol (B and E) and binding was competed by both cholesterol (B, ▲) and 25-hydroxycholesterol (B, △) but not epicholesterol (B, ■). Therefore, the sterol binding characteristics of OSBP are not influenced by the presence of amino acids 311–408 (compare Figs. 6B with 3D).

We next constructed an OSBP that had an internal deletion between amino acids 77 and 310 (OSBPΔ77–310, Fig. 6C) and compared the 25-hydroxycholesterol (D) and cholesterol (E) binding characteristic with OSBP311–809. Whereas OSBPΔ77–310 (D, □) and OSBPΔ77–310 (D, ▲) both bound 25-hydroxycholesterol equally well, cholesterol binding to OSBPΔ77–310 was reduced (E, ▲) compared with OSBP311–809 (E, □). This suggests that the glycine-alanine rich domain between amino acid 1 and 87 (Gly-Ala domain) reduces cholesterol binding to the sterol-binding domain without influencing 25-hydroxycholesterol binding.

We explored further the affect of the Gly-Ala domain on cholesterol binding. First, we constructed a mutant OSBP that lacked the PH domain (OSBPΔ87–182, Fig. 7D). Whereas this construct avidly binds 25-hydroxycholesterol (A, ▲), we could not detect any specific binding of cholesterol (A, □). In the same experiment, OSBP311–809 (A, ■) bound cholesterol. Thus, even though the Kd for 25-hydroxycholesterol binding to OSBPΔ87–182 averaged 6 nM, we could not detect specific binding of cholesterol (Fig. 7D and supplemental Figs. S1 and S3). On the other hand, removing both the PH domain and the Gly-Ala domain restored (OSBP181–809) cholesterol binding (Fig. 7B, ▲ and C). These experiments suggest that the first 87 amino acids of OSBP modulate cholesterol binding to the sterol-binding site without affecting oxysterol binding to this site.
FIGURE 5. Full-length OSBP binds both cholesterol and 25-hydroxycholesterol. Experiments were carried out as described in the legend to Fig. 3. A, constant amount of each lysate was incubated in the presence of the indicated concentration of 25-OH-[3H]-cholesterol for 2 h at room temperature, and purified by nickel chromatography. Specific binding was calculated by subtracting the [3H] associated with the control lysate. B, same protocol was used to measure [3H]cholesterol binding to the full-length OSBP. As a negative control, we measured [3H]cholesterol binding to OSBP1–409. C, diagram of full-length OSBP with the average estimated binding affinity of 25-hydroxycholesterol and cholesterol. A one-way ANOVA followed by multiple comparisons using Fisher’s Least-Significant-Difference Test shows that the binding of sterol to 1–809 is statistically significant (supplemental Fig. S1). D, same sterol binding protocol was used to measure 25-OH-[3H]-cholesterol binding to two mutated full-length OSBPs (Y298S and Y458S). Kd for binding of Y298S was 15.2 ± 1.2 nM. E, full-length OSBP was incubated in the presence of 60 nM [3H]-cholesterol plus the indicated concentration of unlabeled 25-hydroxycholesterol for 2 h at room temperature. The amount of specific [3H]-cholesterol bound was measured. We used immunoblotting to confirm that each sample within an experiment had the same amount of expressed OSBP constructs.
Client Enzymes HePTP/PP2A Bind to the C Terminus of OSBP—The above experiments indicate that amino acids 1–310 regulates sterol binding to amino acids 409–459. We next wanted to localize the HePTP/PP2A binding region of the molecule. Cholesterol is a necessary co-factor in the assembly of the OSBP-HePTP/PP2A complex (1). Therefore, we used our standard co-expression assay to test the ability of 1–473 and 408–809, which both bind cholesterol (Figs. 4B and 3B, respectively), to form a complex with HePTP/PP2A (Fig. 8A). Only OSBP_{408-809} bound both enzymes (lane 2). This is the end of the molecule common to all ORPs, which raises the possibility that HePTP/PP2A might bind to all members of the OSBP family. A cDNA coding for amino acid 514–950 of ORP1 (designated ORP1S) was constructed, which codes for a protein that is 41% identical and 54% similar to OSBP_{408-809}. We compared the ability of OSBP_{408-809} and ORP1S to form a complex with HePTP/PP2A (Fig. 8B). Whereas OSBP_{408-809} efficiently formed a complex (Fig. 8B, lane 2) when co-expressed in cells with HePTP, ORP1 did not form a complex even though immunoblotting indicated it was expressed at a higher level than OSBP_{408-809} (Fig. 8B, compare lanes 1 and 2). We conclude that the binding site(s) for the OSBP client enzymes HePTP and PP2A is between amino acids 408 and 809.
The crystal structure of Osh4 (Fig. 2) reveals a coiled-coil domain between amino acids 318 and 353 that stretches across the backbone of the β-barrel, sterol pocket (14). PAIRCOIL (19) predicts that OSBP has a similar coiled-coil domain between amino acids 732 and 761, which is in the same relative position as the Osh4 coiled-coil region (Fig. 2).

A coiled-coil domain within Osh7p, which is one of the seven yeast ORPs, mediates its sterol-regulated interaction with VPS4p (20). This raises the possibility that coiled-coil domains are common sites of sterol-dependent interaction in ORPs. If so, then an OSBP_{408–809} lacking the coiled-coil domain may not interact with HePTP or PP2A. We constructed a cDNA coding for an OSBP_{408–809} that lacks most of the coiled-coil domain (OSBP_{Δ732–760}) and compared the ability of this construct and OSBP_{408–809} to interact with the two phosphatases (Fig. 8C). Whereas the OSBP_{408–809} bound both HePTP and PP2A (lane 1), OSBP_{Δ732–760} only bound PP2A (lane 2). This suggests that there are separate binding sites for the two enzymes on OSBP and that HePTP binding depends on the coiled-coil domain.
This study confirms and extends our previous proposal that OSBP has a major function in connecting sterol metabolism to critical cell signaling pathways that regulate the cholesterol level of cellular membranes. The results do not preclude the possibility that OSBP has a direct function in intramembrane lipid traffic, but do clarify how it participates in cell signaling.

A remarkable feature of OSBP is its ability to bind both cholesterol and a variety of oxysterols (supplemental Fig. S3). Crystallographic analysis of Osh4 reveals in part how OSBP achieves this feat; bound sterol appears to sit in a β-barrel pocket with its side groups interacting with conserved amino acids through water molecules bound to the sterol (14). Thus, the pocket accommodates lipids with a general sterol shape bound to water. The crystal structure also reveals that the sterol in the pocket is inaccessible from the outside because a 28 amino acid long “lid” caps the opening. The lid appears to be highly flexible because it is not visible in the structure unless Osh4 and sterol are co-crystallized. This suggests the lid is a dynamic region that is capable of controlling the entrance and exit of sterols from the pocket.

**Localization of the Sterol Binding Site**—With this as background, we set out to identify the sterol binding site in OSBP by studying the binding characteristics of various mutant OSBPs in solution. Based on our analysis, OSBP appears to contain a single 51-amino acid long sterol-binding site in the middle of the molecule encompassing amino acids 408–459. The binding characteristics of amino acid 408–459 are similar regardless of whether it is attached to the C-terminal (OSBP 408–809) or the N-terminal (OSBP 1–473) ends of OSBP. Moreover, competition studies indicate that epicholesterol, an optical isomer of cholesterol, is unable to bind. Finally, changing tyrosine 458 to serine 458 in OSBP abolishes specific 25-hydroxycholesterol and cholesterol binding, which indicates this residue is critical for sterol binding. Alignment of Osh4 with OSBP shows that OSBP 408–459 overlaps the lid region of Osh4. Therefore, in addition to its lid function amino acids 408–459 is the primary sterol-binding site in the molecule.

Despite compelling evidence that amino acid 408–459 is the sole sterol-binding site in OSBP, competition experiments with cholesterol and 25-hydroxycholesterol show that the interaction of these two classes of sterols with this site is complex. We previously reported that 25-hydroxycholesterol poorly competed for [³H]cholesterol binding to wild type OSBP (1). We repeated these experiments with our standard binding assay comparing full-length, recombinant OSBP (Fig. 5E) with OSBP 408–809 (Fig. 3, C and D). Once again we found that 25-hydroxycholesterol poorly inhibited cholesterol binding to full-length OSBP. Yet the truncated OSBP 408–809 bound cholesterol and oxysterol individually, and 25-hydroxycholesterol

![FIGURE 8. Localization of HePTP/PP2A binding site to OSBP 408–809. A. His-myc-tagged versions of OSBP 1–473 or OSBP 408–809 were co-expressed with HA-HePTP in HeLa cells. The cytosol was prepared and OSBP purified by nickel chromatography as previously described. Eluted samples were processed for immunoblotting to detect the indicated proteins. B. His-myc-tagged ORP1S or OSBP 408–809 was co-expressed with HA-HePTP, and isolated from cytosol as described above. The samples were then processed to detect the indicated proteins by immunoblotting. C. His-myc-tagged OSBP 408–809 or OSBP 408–809[Δ732–760] was co-expressed with HA-HePTP, and isolated from cell cytosol as above. The samples were then processed to detect the indicated proteins by immunoblotting.](https://example.com/figure8.png)
effectively competed for cholesterol binding. One explanation for these results is that the N-terminal end of OSBP influences how sterols interact with the centrally positioned sterol-binding site. Pursuing this line of reasoning, we found that deletion of the PH domain abolishes cholesterol binding without affecting 25-hydroxycholesterol binding. Removing both the Gly-Ala-rich and the PH domain, however, restored cholesterol binding (Fig. 7, B–D). This result implies that the Gly-Ala domain, in conjunction with the PH domain, controls cholesterol binding to OSBP without affecting 25-hydroxycholesterol binding. Most (75%) of the 77 amino acids in the Gly-Ala domain are alanine (21), glycine (26), or proline (10). The amino acid composition, therefore, predicts this region of the molecule is unstructured. Previous studies have implicated partially unstructured, flexible amino acid sequences in modulating the binding of nucleotide to Myo1b (21) and retinoic acid to CRABP I (22).

Either the addition of oxysterol (15) or the removal of cholesterol (23) stimulates OSBP accumulation in the Golgi apparatus. This dynamic response to the sterol status of the cell is mediated in part by an interaction between the PH domain in OSBP (24, 25) and both PI4P (phosphatidylinositol phosphorylated at the 4 position) and an unidentified component(s) in Golgi membranes (26). The small GTP-binding protein Arf1 may also be involved. Therefore, without a PH domain OSBP is unable to target to the Golgi apparatus (25). Previously we reported that OSBP_{Δ87–182} is unable to bind HePTP/PP2A in cells (1), which we interpreted to mean that the mutant was defective in targeting to Golgi membranes for cholesterol loading. Now we find that OSBP_{Δ87–182} is unable to bind cholesterol in solution, which could also account for poor HePTP/PP2A binding to OSBP_{Δ87–182}. These results imply that the PH domain has a more general role in OSBP function than simply targeting it to the Golgi apparatus. The inability of recombinant OSBP_{Δ87–182} to bind cholesterol in vitro is restored by removing the Gly-Ala-rich region (OSBP_{181–809}). One explanation for these results is that the Gly-Ala domain normally regulates cholesterol binding to the sterol-binding domain (OSBP_{408–458}) but removing the PH domain locks it in a position that prevents cholesterol binding. Whatever the explanation, removing the PH domain unmask a regulatory role for the Gly-Ala domain that had not previously been detected and, in addition, reveals a possible function for the PH domain beyond simply targeting OSBP to the Golgi apparatus.

HePTP and PP2A Binding Sites Contained within OSBP_{408–809}—Kinetic measurements have determined that among all the enzymes tested, HePTP and PP2A are the best at removing phosphate from Tyr-187 and Thr-185 in pERK1/2, respectively, and that removing phosphate from the tyrosine is necessary for dephosphorylating pThr-185 (27). To better

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**FIGURE 9. A model for how OSBP functions.** A, diagram of OSBP molecule. B, model for how OSBP functions. 1) The PH domain binds to PI4P. 2) Cholesterol is extracted from the membrane by the sterol-binding domain. 3) The sterol binding domain then transfers cholesterol into the β-barrel pocket. 4) With cholesterol in the β-barrel, the OSBP undergoes a conformational change that allows HePTP/PP2A to bind and cooperatively dephosphorylate pERK1/2.
understand how these two phosphatases coordinate the removal of the two phosphates, it is important to identify where they bind OSBP. We first compared the ability of OSBP_1–473 and OSBP_408–809 to bind the two enzymes and found that both bind cholesterol but only OSBP_408–809 interacts with HePTP/PP2A. We then identified a coiled-coil domain in OSBP_408–809 between amino acids 732 and 761 that is required for HePTP binding. PP2A still bound OSBP_A732–761, which indicates that it does not bind either HePTP or the coiled-coil domain. In yeast, the sterol-regulated interaction between Vps4p and Osh7p is also dependent on a coiled-coil domain in Osh7p (20), only in this case ergosterol inhibits binding. The coiled-coil domain in both ORP1S and OSBP appears to be on the external surface of the β-barrel. HePTP, as well as its family members, also have exposed coiled-coil like domains, which raises the possibility that binding to OSBP is mediated by an intermolecular interaction between coiled-coil domains on each molecule.

What remains to be determined is how PP2A binds OSBP. PP2A is a heterotrimeric enzyme consisting of an A subunit rich in HEAT motifs, a regulatory B subunit also rich in HEAT-like α-helices and a catalytic subunit (28). OSBP interacts either directly or indirectly with all three subunits (1). Recent structural analysis of PP2A (29, 30) suggests either the A or the B subunit probably anchors it to OSBP. PP2A must bind in such a way that the catalytic subunit can act jointly with HePTP to dephosphorylate pTyr-187 and pThr-185 in pERK1/2 in pERK1/2. Stringent cooperation between the two molecules most likely is required because the phosphate on pTyr-187 must be removed by HePTP before PP2A can dephosphorylate pThr-185 (27). In fact, the interaction between OSBP, PP2A, and HePTP is so intimate that simply inhibiting HePTP in the OSBP-HePTP/PP2A complex with vanadate stimulates dephosphorylation of OSBP by bound PP2A (1), and each phosphatase-specific inhibitor blocks the activity of the other phosphatase only when both are in the complex (3).

A Working Model—The results of this study supports the view that OSBP functions as a scaffolding protein for HePTP/PP2A (designated PAOBP (PTPPBS-PP2A-OSBP phosphatase), and suggests a model for how it functions (Fig. 9). The central element of this model is the sterol-binding domain (orange). Although able to bind both cholesterol and oysterols, most likely the domain can only obtain cholesterol from cholesterol-rich membranes such as those found in the trans Golgi apparatus (Fig. 9B, 1 and 2). Extracting cholesterol should be most efficient when OSBP is docked at the membrane, which may be mediated by the PH domain. Therefore, OSBP is targeted to the Golgi apparatus through an interaction between its PH domain and both PI4P and unidentified cofactors in the Golgi. Once docked, the OSBP sterol-binding domain extracts cholesterol and moves it into the β-barrel pocket (Fig. 9B, 3). In this model, the sterol-binding domain is an extractor that removes cholesterol from the membrane, a shuttle that moves it into the β-barrel, and a lid that holds the sterol in the β-barrel pocket. Once cholesterol is in the pocket, the β-barrel undergoes a conformational change that favors the binding of HePTP and PP2A to the C terminus (Fig. 9B, 4). The conformational shift in the shape of the N terminus causes OSBP to detach from the membrane and, at the same time, move the Gly-Ala domain closer to the sterol-binding domain, where it modulates sterol binding.

One of our key observations about PAOBP is that both cholesterol depletion and the addition of 25-hydroxycholesterol cause disassembly of the complex. Both conditions also cause OSBP to migrate to the Golgi apparatus. Using recombinant protein in bacterial lysates we find that 25-hydroxycholesterol does not compete for cholesterol binding to full-length OSBP. This implies that within the cell cholesterol must be removed from OSBP before 25-hydroxycholesterol can bind. Therefore, OSBP has the ability to sense both the level of cholesterol in a membrane and the presence of damaged cholesterol that might have accumulated as a result of oxidative activities operating locally in the membrane. We postulate, therefore, that membrane bound OSBP is in constant flux with cholesterol. If oxysterols increase, it will replace the cholesterol in OSBP because of its higher affinity thereby inactivating PAOBP. Likewise, PAOBP will become inactivated when the cholesterol level of the membrane falls. Both conditions increase the amount of pERK1/2, which leads to the activation of machinery that supplies new cholesterol to the membrane.

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