Multi-Site Phosphorylation of Oxysterol Binding Protein (OSBP)

Regulates Sterol Binding and Activation of Sphingomyelin Synthesis

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*Running Head: Phosphorylation regulation of OSBP

Abbreviations: CERT, ceramide transport protein; CK1, casein kinase 1; ER, endoplasmic reticulum; FFAT, two phenylalanines in an acidic tract; 25OH, 25-hydroxycholesterol; OSBP, oxysterol binding protein; ORP, OSBP-related protein; PI, phosphatidylinositol; PI(4)P, phosphatidylinositol 4-phosphate; PI(4,5)P2, phosphatidylinositol 4,5-bisphosphate; PH, pleckstrin homology; PKD, protein kinase D; SM, sphingomyelin; TGN, trans-Golgi network; TEM, transmission electron microscopy; VAP-A-A, vesicle-associated-membrane protein-associated protein-A

ABSTRACT

The endoplasmic reticulum (ER)-Golgi sterol transfer activity of OSBP regulates sphingomyelin (SM) synthesis as well as post-Golgi cholesterol efflux pathways. The phosphorylation and ER-Golgi localization of OSBP are correlated suggesting that this modification regulates the directionality and/or specificity of transfer activity. Here we report that phosphorylation on two serine-rich motifs, S381-S391 (Site 1) and S192, S195, S200 (Site 2), specifically controls OSBP activity at the ER. A phosphomimetic of the SM/cholesterol-sensitive phosphorylation Site 1 (OSBP-S5E) had increased in vitro cholesterol and 25-hydroxycholesterol binding capacity, and cholesterol extraction from liposomes, but reduced transfer activity. Phosphatidylinositol 4-phosphate (PI(4)P) and cholesterol competed for a common binding site on OSBP; however, direct binding of PI(4)P was not affected by Site 1 phosphorylation. Individual Site 1 and Site 2 phosphomutants supported oxysterol-activation of SM synthesis in OSBP-deficient CHO
cells. However, a double Site1/2 mutant (OSBP-S381A/S3D) was deficient in this activity and was constitutively co-localized with vesicle-associated-membrane protein-associated protein-A (VAP-A) in a collapsed ER network. This study identifies phosphorylation regulation of sterol- and VAP-A-binding by OSBP in the ER, and PI(4)P as an alternate ligand that could be exchanged for sterol in the Golgi apparatus.

INTRODUCTION

Mammalian oxysterol binding protein (OSBP) and OSBP-related proteins (ORPs) constitute a large eukaryotic gene family characterized by a conserved C-terminal sterol-binding domain (Lehto and Olkkonen, 2003). Full-length OSBP/ORPs also have N-terminal pleckstrin homology (PH) domains that interact with phosphatidylinositol (PI) phosphates (Levine and Munro, 1998; Levine and Munro, 2002) and a two phenylalanines (FF) in an Acidic Tract (FFAT) motifs that bind vesicle-associated-membrane protein-associated protein-A (VAP-A) on the cytoplasmic surface of the endoplasmic reticulum (ER) (Loewen et al., 2003; Wyles et al., 2002). This domain organization suggests that a primary function of OSBP/ORPs is to transfer cholesterol, ergosterol or oxysterols between target membranes, and/or tranduce sterol-dependent signals at these points of contact (Ngo et al., 2010; Prinz, 2007).

Oxysterol binding protein homologues (OSH) in S. cerevisiae catalyze phosphatidylinositot 4,5-bisphosphate (PI(4,5)P2)-dependent ergosterol transfer in vitro and regulate cholesterol-delivery from the ER to plasma membrane (PM) (Raychaudhuri et al., 2006). However, Osh4p and other Osh proteins are also implicated in the regulation of plasma membrane (PM) phosphatidylinositol 4-phosphate (PI(4)P) levels (Stefan et al., 2011), PM sterol distribution (Georgiev et al., 2011) and polarized
endocytosis by mechanisms that evoke sterol sensing activity (Alfaro et al., 2011). The integration of PI(4)P and ergosterol distribution and function by Osh4p could be due to competitive, high affinity binding and transfer of these two ligands (de Saint-Jean et al., 2011). OSBP family members bind cholesterol and oxysterols, and some have been implicated in sterol transfer between organelles (Banerji et al., 2010; Ngo and Ridgway, 2009; Suchanek et al., 2007). ORP9L and OSBP extract and transfer cholesterol between liposome in vitro (Ngo and Ridgway, 2009). Both proteins localize to the ER and Golgi apparatus and influence the post-Golgi distribution of cholesterol in cultured cells, suggesting that they directly or indirectly transfer cholesterol within the early secretory pathway. In the case of OSBP, sterol-transfer between the ER and Golgi apparatus is coupled to activation of ceramide transport protein (CERT) and sphingomyelin (SM) synthesis through increased activity of PI4KIIα, a cholesterol sensitive PI 4-kinase (Banerji et al., 2010; Waugh et al., 2006). On the other hand, OSBP and ORPs are implicated in regulation of diverse signaling pathways that could be the result of sterol-sensing or -transduction activity (Johansson et al., 2007; Lehto et al., 2008; Lessmann et al., 2007; Ngo et al., 2010; Wang et al., 2005).

The ER is cholesterol-poor compared to later secretory compartments indicating that transport proteins must utilize a mechanism to deliver ligands against a concentration gradient. In this regard, OSBP is subject to a rapid phosphorylation cycle that is linked to Golgi-localization and the cholesterol and SM content of cells (Ridgway et al., 1998b; Storey et al., 1998). Rapid depletion of SM in the PM results in dephosphorylation of OSBP and localization to the Golgi apparatus. Similarly, depletion of cholesterol by prolonged inhibition of synthesis caused OSBP dephosphorylation and Golgi localization.
Since SM and cholesterol are essential components of PM rafts, OSBP phosphorylation appears to be dependent on the integrity of these membrane domains. A serine-rich motif situated between the FFAT and sterol binding domain of OSBP (S381, S384 and S387) (Figure 1) is responsive to changes in sterol and SM; S381 is required for subsequent phosphorylation of S384 and S387, indicating these later two sites are phosphorylated by casein kinase 1 (CK1) (Mohammadi et al., 2001). A similar cholesterol and SM-sensitive phosphorylation site in CERT negatively regulates PH domain interaction with PI(4)P in the Golgi apparatus (Kumagai et al., 2007). This serine-rich motif consists of a protein kinase D (PKD) phosphorylation site (S132) (Fugmann et al., 2007) that regulates phosphorylation of 9 adjacent serine and threonine residues by CK1γ2 (Tomishige et al., 2009). Dephosphorylation in the ER by protein phosphatase 2Cε relieves inhibition of the PH domain (Saito et al., 2008). This identified two functional states for CERT; a dephosphorylated form that associates with the Golgi and mediates ceramide transfer from the ER, and an inactive PKD/CK1 phosphorylated form that binds ceramide but does not interact with the Golgi apparatus. The regulation of CERT and OSBP at the level of sterol- and SM-mediated phosphorylation, and the sterol-depend activation of CERT by OSBP (Banerji et al., 2010; Perry and Ridgway, 2006), demonstrates that this functional pair have an important role in regulating membrane homeostasis in the secretory pathway.

To understand how phosphorylation regulates OSBP we undertook a functional analysis of phosphorylation sites adjacent to the sterol binding (Site 1) and PH (Site 2) domains. Results with phospho-mimetic mutants indicate that the VAP-A- and sterol-binding phase of OSBP at the ER is regulated by phosphorylation at these two sites, and
is functionally linked to sterol-dependent activation of SM synthesis in the Golgi apparatus. We also demonstrate that the PI(4)P binding activity of OSBP is competitive with cholesterol but insensitive to phosphorylation of Site 1, indicating involvement in a Golgi-specific phase of the transfer cycle.

RESULTS

**OSBP Site 1 phosphorylation specifically increases sterol-binding activity**

OSBP is phosphorylated between the sterol binding and FFAT domains (Site 1), as well as a PKD site at S242 (Mohammadi et al., 2001; Nhek et al., 2010). Recent phosphoproteome studies have extended Site 1 to include phosphorylation at T379, S388 and S391, constituting a large CK1 motif that is activated by phosphorylation of S381 (Figure 1) (Dephoure et al., 2008; Gauci et al., 2009; Zahedi et al., 2008). These studies also identified a cluster of 3 phosphoserine residues (positions 192, 195 and 200, designated Site 2) in a potential casein kinase 2 motif next to the PH domain. To determine how phosphorylation at Site 1 and Site 2 regulates OSBP activity, serine and threonine residues were mutated to mimic fully dephosphorylated or phosphorylated species (summarized in Figure 1). In the case of Site 1, OSBP-S381A prevents phosphorylation of downstream serine residues and mimics a dephosphorylated state with increased mobility on SDS-PAGE, while introduction of glutamine residues simulates a phosphorylated state with reduced mobility on SDS-PAGE (Figure 2, 5 and (Mohammadi et al., 2001).

To establish whether phosphorylation of Site 1 affects OSBP activity *in vitro*, OSBP and OSBP-S381A, -S3E and -S5E were expressed and purified from SF21 cells and assayed for sterol-binding, -extraction and -transfer activities (Figure 2). The
migration of each protein on SDS-PAGE was consistent with its proposed phosphorylation state; S381A migrated as a dephosphorylated species, while OSBP-S3E and -S5E mimicked a phosphorylated state with reduced mobility (Figure 2A). Wild-type OSBP expressed in Sf21 cells is dephosphorylated at Site 1 as indicated by co-migrated with OSBP-S381A. OSBP displayed saturable binding of \( [^3\text{H}]25\)-hydroxycholesterol (25OH) (Kd 28±5 nM; Bmax 0.63±0.05) that was similar to OSBP-S381A (Kd 51±12 nM; Bmax 0.68±0.11) and OSBP-S3E (Kd 58±14; Bmax 0.49±0.15) (Figure 2B). The Kd for oxysterol binding to OSBP-S5E (35±9 nM) was similar to wild-type and the other mutants but binding capacity (Bmax 2.0±0.26) was increased 3-fold. Specific binding of \([^3\text{H}]\)cholesterol by OSBPs was saturable but calculated binding constants were inconsistent due to non-linear Scatchard plots (Figure 2C). However, it is evident that maximal cholesterol binding by OSBP-S5E was also increased >2-fold compared to OSBP and the other phospho-mutants. The rate of sterol dissociation was determined by incubating \([^3\text{H}]25\text{OH}\)-loaded OSBP or OSBP-S5E in buffer containing unlabeled 25OH at 20°C and measuring the appearance of radiolabelled oxysterol in the supernatant (Figure 2D). Under these conditions, \([^3\text{H}]25\text{OH}\) exchanged more slowly from OSBP-S5E compared to wild-type OSBP.

We next determined whether OSBP-S5E had altered cholesterol extraction and transfer activity using liposomal substrates. Extraction was measured by incubating increasing amount of OSBPs with liposomes containing 1% \([^3\text{H}]\)cholesterol and measuring the appearance of radiolabel in the supernatant after donor liposome removal (Figure 2E). Similar to results with detergent-dispersions of cholesterol (Figure 2C), OSBP-S5E extracted 2-fold more \([^3\text{H}]\)cholesterol from liposomes compared to OSBP and
OSBP-S381A. The effect of Site 1 phosphorylation on cholesterol transfer was determined using a modified assay in which OSBPs was first incubated with donor liposomes to initiate $[^{3}H]$cholesterol extraction followed by addition of acceptor liposomes (Figure 2F). The percentage of $[^{3}H]$cholesterol transferred by all OSBPs to acceptor liposomes containing PI was negligible but was stimulated by the presence of 2 mol% PI(4)P. Although OSBP-S5E extracted twice as much cholesterol as OSBP and OSBP-S381A (Figure 2E), the percentage transferred to acceptor liposomes with PI(4)P was similar, indicating less efficient delivery of bound cholesterol. Transfer by a OSBP PH domain mutant (RR/EE) that does not interact with PI(4)P was reduced by 75% confirming previous results that the PH domain has a stimulatory role in transfer (Ngo and Ridgway, 2009). Like Osh4p (de Saint-Jean et al., 2011), cholesterol transfer by OSBP is stimulated by PI(4)P in acceptor liposomes but the N-terminal PH domain also has a positive role.

PI(4)P occupies the Osh4p sterol-binding pocket with its 4-phosphate residue associated with a phylogenetically conserved histidine pair, raising the possibility that other OSBP/ORPs bind both sterols and PI(4)P (de Saint-Jean et al., 2011). Thus we used competition and direct binding assays to determine if OSBP binds PI(4)P, and whether Site 1 mutations affect this binding activity. First, OSBP extraction of $[^{3}H]$cholesterol was assayed using liposomes that contained increasing amounts of unlabeled PI, PI(4)P or PI(4,5)P$_2$ (Figure 3A). Inclusion of PI or PI(4,5)P$_2$ in donor liposomes did not inhibit $[^{3}H]$cholesterol extraction compared to liposomes without PIPs. In contrast, inclusion of PI(4)P caused a 75% reduction in $[^{3}H]$cholesterol extraction. To exclude the possibility that this resulted from OSBP sequestration by PI(4)P containing
liposomes, the distribution of OSBP between the supernatant and pellet fraction of the assay shown in Figure 3A was subject to SDS-PAGE and protein staining (Figure 3B). It is evident that concentrations of PI(4)P that inhibited [3H]cholesterol to OSBP did not increase OSBP association with donor liposomes in the pellet fraction.

To verify the results from completion assays shown in Figure 3A, direct binding and extraction of radiolabelled PI and PI(4)P by OSBP was measured (Figure 3C and D). OSBP extracted significantly more [32P]-labeled PI(4)P from liposomes compared to [3H]PI (Figure 3C). [32P]PI(4)P extraction by OSBP was dose-dependent but became non-linear after 50 pmol (Figure 3D). Interestingly, OSBP-S5E extracted similar amounts of [32P]PI(4)P compared to wild-type and OSBP-S381A indicating that phosphorylation of Site 1 specifically stimulates sterol binding activity. Thus in addition to binding PI(4)P via the PH domain, OSBP has an additional PI(4)P binding site that is competitive with cholesterol.

**OSBP site 1 phosphorylation does not affect oxysterol-activation of SM synthesis**

Since OSBP-S5E has increased sterol binding activity, and oxysterol and cholesterol binding is known to enhance OSBP association with the Golgi apparatus (Lagace et al., 1999), we tested whether OSBP-S5E and other Site 1 phospho-mutants have altered membrane binding activity and Golgi localization (Figure 4). PH-domain-dependent interactions of OSBPs were assessed by binding to phosphorylated PIs on nitrocellulose and in liposomes, although the later assay is complicated by the potential for PI(4)P to be bound and extracted by the sterol-binding domain (Figure 3). Wild-type OSBP and the Site 1 phospho-mutants had similar, preferential binding to PI(4)P that was immobilized on nitrocellulose filters (Figure 4A). Similarly, OSBP binding to liposomes containing
10 mol% PI(4)P or PI(4,5)P₂ was unaffected by Site 1 mutations, although this assay indicated a binding preference for PI(4,5)P₂ (Figure 4B). To assess interaction of OSBP Site 1 mutants with the Golgi apparatus, N-terminal mCherry fusions were transiently expressed in CHO cells and cellular localization was determined by immunofluorescence microscopy in the presence and absence of 25OH (Figure 4C). OSBP and OSBP-S5E were primarily in a diffuse cytoplasmic/ER compartment in the absence of 25OH but became strongly localized to the perinuclear Golgi compartment in cells exposed to 25OH. OSBP-S381A had a slightly more pronounced perinuclear distribution in the absence of 25OH but translocated to the Golgi apparatus in oxysterol-treated cells. Untagged OSBP mutants showed similar behavior in response to 25OH treatment (result not shown). Although the Site 1 phospho-mutants did not have altered ER-Golgi localization, the proximity of Site 1 to the FFAT motif suggests that phosphorylation could increase negative charge in this region and influence VAP-A interaction. To assess this in vitro, increasing amounts of recombinant OSBP Site 1 mutants were incubated with GST-VAP-A and interaction was determined by pull-down and SDS-PAGE (Figure 4D). Binding of OSBPs by GST-VAP-A was relatively linear over the chosen concentration range, and there was no significant difference in the amount of OSBP-S381A or -S5E associated with GST-VAP-A compared to wild-type OSBP. These in vitro experiments show that Site 1 phosphorylation specifically enhances sterol-binding capacity without affecting the FFAT and -PH domain interactions of OSBP.

Sterol-mediated translocation of OSBP to the Golgi apparatus activates CERT-dependent ceramide delivery to the Golgi apparatus for conversion to SM (Perry and Ridgway, 2006). Reconstitution of SM synthesis (measured by [³H]serine incorporation)
in OSBP-deficient CHO cells by cDNA transfection can be used as a functional readout to assess the influence of OSBP mutations on this pathway. Using this approach, OSBP Site 1 mutants were tested for their ability to restore 25OH-activated SM synthesis in CHO cells depleted of endogenous OSBP (Figure 5). Compared to mock transfected cells, OSBP expression in deficient CHO cells did not affect synthesis under unstimulated conditions but restored a 2.5-fold activation SM synthesis by 25OH (Figure 5A). Expression of OSBP-S381A or the phospho-mimics OSBP-S3E or -S5E also restored SM synthesis to a level that was not significantly different from wild-type. T379 phosphorylation was also evaluated as a potential regulatory site of SM synthesis. OSBP-T379A migrated as a doublet on SDS-PAGE (Figure 5C) indicating that Site 1 phosphorylation is intact and phosphorylation of S381 alone triggers phosphorylation of the adjacent four serine residues. Expression of OSBP-T379A or a mutant with all 6 threonine and serine residues changed to glutamate (OSBP-TS6E) restored SM synthesis (Figure 5A). \[^3\text{H}\]Serine incorporation into ceramide in mock and OSBP-transfected CHO cells was similar under control and 25OH treated conditions (Figure 4B). Collectively this suggests that phosphorylation at Site 1 is sufficient to enhance sterol binding but not subsequent steps in OSBP regulation of SM synthesis at the Golgi apparatus.

**OSBP is phosphorylated at Site 2 adjacent to the PH domain**

Phosphoproteome studies identified additional phosphoserines at positions 192, 195 and 200 that are embedded in a negatively changed region adjacent to the PH domain (Figure 1). To confirm phosphorylation at Site 2, OSBP and single or double Site 1/Site 2 mutants that prevent phosphorylation (S3A and S381A) were transiently transfected into
CHO cells and phosphorylation status was monitored by immunoblotting and phosphopeptide analysis (Figure 6). OSBP-S3A had similar mobility as OSBP on immunoblots and reduced $^{32}$PO$_4$ incorporation into the lower band but not the upper band, indicating that Site 1 was unaffected (Figure 6A). OSBP-S381A lacked the upper phosphorylated species corresponding to Site 1 phosphorylation but had similar incorporation of $^{32}$PO$_4$ into the lower band, suggesting that Site 2 phosphorylation was unaffected. The double mutant lacked the upper band and had reduced incorporation into the lower species. Tryptic digestion of immunoprecipitated OSBP and 2-dimensional mapping revealed a single phosphopeptide (indicated by arrows) containing Site 2 phosphoserine residues that was absent in both S3A and S381A/S3A mutants (Figure 6B). The S3A mutation did not affect $^{32}$PO$_4$ incorporation into the three phosphopeptides that constitute this site (indicated by asterisks, Figure 6B). The phosphopeptides remaining in OSBP-S381A/S3A could be due to phosphorylation of S242 by PKD and/or S353 by an unknown kinase (unpublished results). This confirms that Site 2 is phosphorylated in CHO cells but it does not appear to be required for Site 1 phosphorylation and vice versa.

**A Site 1/Site 2 double mutant is non-functional and aggregates the ER**

Restoration of 25OH-activated SM synthesis in OSBP-depleted CHO cells was used to assess the role of Site 2 in OSBP activity (Figure 7). Expression of the Site 2 phosphorylation mutants S3A and S3D restored 25OH-activated [³H]serine incorporation into SM and ceramide to the level of cells expressing OSBP (Figure 7A). Immunoblots of total cell lysates further established that Site 2 mutations did not affect the slower migrating Site 1 phospho-species (Figure 7C). Immunofluorescence localization
experiments confirmed that OSBP-S3A and –S3D localization was similar to wild-type in control and 25OH-treated cells (Supplemental Figure 1).

Since individual phosphorylation site mutations did not affect OSBP function, we decided to test a series of four Site 1/Site 2 double mutants (Figure 7B and D). Three of these mutants (S381A/S3A, S5E/S3D and S5E/S3A) restored a 2.5-fold activation of SM synthesis by 25OH without affecting basal synthesis. In contrast, expression of OSBP-S381A/S3D failed to restore oxysterol-activation of SM synthesis without affecting basal SM or ceramide synthesis. OSBP-S381A/S3D was highly expressed compared to mock cells, in which RNAi silencing is >95% effective, but was only 20-25% compared to the other double mutants (Figure 7D).

To determine why OSBP-S381A/S3D was defective with respect to sterol-activation of SM synthesis, its intracellular localization was examined by immunofluorescence and transmission electron microscopy (TEM) in OSBP-depleted CHO cells (Figure 8). Site 1/Site 2 double mutants that restored SM synthesis in CHO cells (Figure 7) showed the expected translocation from an ER/cytoplasmic compartment to the Golgi apparatus in response to oxysterol treatment (Supplemental Figure 1). Interestingly, OSBP-S381A/S3D was exclusively localized to unusual filamentous structures around the nucleus of CHO cells (Figure 8A). These structures were absent in adjacent non-expressing cells, unaffected by 25OH treatment and co-localized with VAP-A, which is normally present in an extended reticular network. These aggregated structures did not localize with the trans-Golgi enzyme PI4KIIIβ, but cells expressing OSBP-S381A/S3D had a dispersed PI4KIIIβ staining pattern (Figure 8B). A similar pattern of ER aggregation and co-localization of OSBP-A381A/S3D with VAP-A was
observed in HeLa cells (results not shown). To determine if aggregation of the ER by OSBP-S381A/S3D was due to interaction with VAP-A, we mutated the FFAT motif (FF-AA), expressed the triple mutant in CHO cells and determined its location relative to endogenous VAP-A (Figure 8C). Unlike the double phospho-mutant, OSBP-S381A/S3D/FF-AA was dispersed in the cytoplasm or in small punctate structures and did not co-localize with VAP-A or cause its aggregation. Interestingly, OSBP-S381A/S3D/FF-AA did not localize to the Golgi apparatus when cells were treated with 25OH suggesting a non-functional PH domain.

To better visualize ultrastructural changes to the ER induced by OSBP-S381A/S3D, CHO cells expressing the mutant were analyzed by TEM (Fig 8D-I). CHO cells expressing OSBP displayed normal organelle morphology with many ribosome-studded ER tubules (indicated by arrows, Figure 8D). In contrast, CHO cells expressing OSBP-S381A/S3D contained extended aggregates of membrane tubules that were close to the nucleus and devoid of ribosomes (Figure 8E-G). Cells containing these ER aggregates were also devoid of normal ER tubules elsewhere in the cell. Immunostaining for OSBP-S381A/S3D showed the presence 5 nm colloidal gold particles in areas enriched in tubule aggregates (Figure 8H and I). Thus OSBP-S381A/S3D caused the ER to collapse into filamentous bundles and dispersed the Golgi apparatus, perhaps contributing to its poor expression compared to other mutants (Fig. 7D).

To assess whether the co-localization of OSBP-S381A/S3D and VAP-A observed by immunofluorescence was the result of enhanced physical interaction, and whether other phospho-mutants might display altered binding, interaction with VAP-A was assessed by co-immunoprecipitation from OSBP transfected CHO cells (Figure 9).
Detergent extracts from cells expressing OSBP Site 1 and Site 2 phosho-mutants were immunoprecipitated using a VAP-A antibody and probed for OSBP. There were no reproducible differences in the amount of single OSBP mutants co-immunoprecipitated with VAP-A under control or 25OH treated conditions (Figure 9A). Compared to wild-type, OSBP-S381A/S3A, -S5E/S3D and -S5E/S3A double mutants had similar levels of expression and interaction with VAP-A (Figure 9B). Despite reduced expression of OSBP-S381A/S3D, it showed similar levels of co-immunoprecipitation with VAP-A compared to the other double mutants. When quantified relative to input, approximately 6-fold more OSBP-S381A/S3D was co-immunoprecipitated with VAP-A compared to the other three double mutants (Figure 9C). This further shows that OSBP-S381A/S3D contributes to ER aggregation due to enhanced interaction with VAP-A multimers.

**DISCUSSION**

The differential localization of OSBP between the ER and Golgi apparatus in response to exogenous and endogenous sterol ligands, and resultant effects on sterol responsive activities in the Golgi apparatus, suggests that OSBP transfers cholesterol and/or oxysterols between these organelles. This activity can be partially reconstituted *in vitro* if cholesterol extraction and transport is down a concentration gradient. This is unlikely to occur *in vivo* since the ER is cholesterol-poor relative to the Golgi and other organelles (Ikonen, 2008). In this study we identify two factors that could facilitate sterol loading and transport to the Golgi apparatus by OSBP; phosphorylation on two serine-rich motifs (Sites 1 and 2) that control sterol-binding and interaction with VAP-A in the ER, and competition for sterol binding by PI(4)P. OSBP phosphorylation regulates the ER-
specific sterol/VAP-A binding but not the binding of PI(4)P, a ligand that is highly enriched in the Golgi apparatus where it could exchange with cholesterol.

Site 1 phosphorylation is initiated at S381, which triggers phosphorylation of four adjacent serine residues by a CKI-like activity (Mohammadi et al., 2001). The organization of Site 1 is reminiscent of the serine-rich motif in CERT, which is initiated by PKD phosphorylation and sensitive to cellular cholesterol and SM content (Kumagai et al., 2007). However, OSBP-S381 is not phosphorylated by PKD nor is PKD phosphorylation of S242 involved in Site 1 phosphorylation (results not shown). While the PH and FFAT domain activity was unaffected by Site 1 mutations, introducing a constitutive negative charge in OSBP-S5E, which is proximal to the α-helical lid of the sterol binding domain, caused a significant increase in the maximal binding of aqueous dispersions of 25OH without significant affecting the apparent Kd. The partially phosphorylated mimetic OSBP-S3E had similar sterol binding properties as OSBP and OSBP-S381A indicating that maximal phosphorylation of Site 1 is necessary to increase sterol binding. The cholesterol binding capacity of OSBP-S5E for cholesterol dispersions was similarly increased relative to dephospho-mimics but dissociation constants could not be reproducibly measured due to irregular binding curves, possible related to poor ligand solubility and non-equilibrium conditions that were employed to avoid protein inactivation. OSBP-S5E also extracted more cholesterol from liposomes compared to dephospho-mimics, but the extracted cholesterol was not efficiently transferred to acceptor liposomes. This implies that phosphorylation at Site I increases the initial capacity of OSBP to bind sterols at a donor membrane, and subsequent dephosphorylation facilitates the release of the sterol ligand to an acceptor membrane. In
intact cells, $^{32}$PO$_4$ pulse-chase experiments showed that phosphorylation of Site 1 has a half-life of approximately 20 min (Ridgway et al., 1998a). This is consistent with a dynamic phosphorylation cycle at this site that, in conjunction with other protein/lipid interactions and phosphorylation sites, regulates sterol binding and release.

Site 1 phosphorylation is correlated with changes in OSBP localization, particularly in response SM and cholesterol metabolism and content (Mohammadi et al., 2001; Ridgway et al., 1998b). However, individual Site 1 mutations did not affect \textit{in vitro} interactions with VAP-A or PI(4)P or the distribution of OSBP between the ER and Golgi apparatus in CHO cells, indicating it is not sufficient to induce these changes and other variables are involved. Site 2 was of interest in this regard since it encompasses three serine residues in a glutamate/aspartate-rich region located next to the PH domain. $^{32}$PO$_4$–labeling and peptide mapping of Site1 and Site 2 mutants indicated no interdependence between the sites and, similar to Site 1, individual Site 2 phosphomutants were normal with respect cellular localization and restoration of 25OH-activated SM synthesis. However, expression of a mutant mimicking de-phosphorylation of Site 1 and phosphorylation of Site 2 (OSBP-S381A/S3D) collapsed the peripheral ER into membrane bundles around the nucleus, and strongly co-localized and associated with VAP-A. A similar ER morphology was observed in cells expressing an OSBP PH domain mutant (W174A) (Wyles and Ridgway, 2004) and Nir proteins, PI binding/transfer proteins that interact with VAP-A via FFAT domains (Amarilio et al., 2005; Peretti et al., 2008). In these instances, enhanced interaction with VAP-A causes cross-linking of ER tubules and collapse of the reticular network into membrane bundles and whorls. The similarity between OSBP W174A and OSBP-S381A/S3D with respect
to ER perturbation and VAP-A interaction suggests that the latter mutant also has a nonfunctional PH domain. In support of this conclusion, mutation of the FFAT domain in OSBP-S381A/S3D prevented interaction with VAP-A and collapse of the ER, but the mutant did not regain the ability to localize to the Golgi in the presence of 25OH. This suggests a phosphorylation state that causes reduced cholesterol binding activity, increased VAP-A binding and reduced affinity for PH domain ligands in the Golgi apparatus. Based on the normal activity of other single and double Site 1/Site 2 mutants, subsequent changes in the phosphorylation status of either Site 1 or 2, by as yet unknown kinases and/or phosphatases, would disengage OSBP from the ER and promote interaction with the Golgi apparatus.

OSBP homologues in diverse phyla are distinguished by two conserved histidine residues (H522 and H523 in human OSBP) situated at the entrance of the sterol binding pocket (Im et al., 2005; Lehto and Olkkonen, 2003). This histidine pair is essential for Osh4p function but dispensable for *in vitro* sterol binding and transfer activity (Im et al., 2005; Raychaudhuri et al., 2006). Instead, these histidine residues form an essential contact with the 4-phosphate residue of PI(4)P that mediates competitive binding with dehydroergosterol (de Saint-Jean et al., 2011). This suggested that Osh4p transport of ergosterol to PI(4)P-enriched compartments is coupled to exchange and transport of PI(4)P in the opposite direction. This model is particularly suited to OSBP, which could transport cholesterol against a gradient to the PI(4)P-enriched trans-Golgi/TGN in conjunction with PI(4)P transport in the opposite direction. Initial *in vitro* experiments lend support to this model; cholesterol extraction from liposomes by OSBP was specifically competed by PI(4)P, and radiolabelled PI(4)P was extracted by OSBP from
cholesterol-free liposomes. Based on the percent extraction of cholesterol and PI(4)P from liposomes by increasing amounts of OSBP (compare Figure 2E with 3D), the relative affinity of OSBP for PI(4)P and cholesterol is comparable. Interestingly, the increased sterol binding activity of OSBP-S5E toward cholesterol was not evident with PI(4)P. This indicates that phosphorylation events that affect the unique electrostatic and hydrogen-bonding contacts for sterols and PI(4)P could regulate an organelle-specific interaction with these ligands.

To test whether PI(4)P stimulated the transfer of cholesterol to acceptor liposomes we used a modified assay that involved pre-extraction of cholesterol from donor liposomes. In this context, PI(4)P stimulated the transfer of cholesterol by OSBPs suggesting an exchanged-based mechanism similar to Osh4 (de Saint-Jean et al., 2011). However, this is not the only explanation since efficient cholesterol transfer by OSBP also required its N-terminal PH domain (Figure 2F) (Ngo and Ridgway, 2009). The PH domains could facilitate exchange by targeting OSBP to PI(4)P-enrich membranes or regulate ligand transfer by inter/intramolecular interactions.

This study provides a new perspective on OSBP activity in the ER-Golgi secretory pathway through identification of serine phosphorylation motifs that regulate the ER-specific phase of the OSBP transfer cycle involving VAP-A and sterol binding. The subsequent release of sterols in the Golgi apparatus could involve ligand exchange with PI(4)P. This exchange could be driven by OSBP-dependent activation of PI(4)P synthesis by the sterol-regulated PI4KII\(\alpha\) (Banerji et al., 2010). Alternatively, PKD could also regulate OSBP at the Golgi apparatus via direct phosphorylation (Nhek et al., 2010) or activation of PI(4)P synthesis by PI4KIII\(\beta\) (Hausser et al., 2005).
MATERIALS AND METHODS

Materials

Cholesterol and 25OH were purchased from Steraloids. $[^3]H$Cholesterol, $[^3]H$25OH, $^{32}$PO$_4$, $[^3]H$PI and $[^3]H$serine were from Perkin-Elmer (Waltham, MA). PI(4)P (dipalmitoyl and bovine brain) and PI(4,5)P$_2$ (dipalmitoyl) were from Echelon (Salt Lake City, UT). Phosphatidylcholine (PC) was purchased from Avanti Polar Lipids (Alabaster, AB). OSBP polyclonal and monoclonal antibodies were previously described (Mohammadi et al., 2001; Ridgway et al., 1992). A VAP-A-specific antibody was previously described (Wyles et al., 2002). A colloidal gold-conjugated secondary antibody was purchased from Sigma-Aldrich (St. Louis, MO). Alexafluor488- and 594-conjugated secondary antibodies, and Baculo-Direct expression system were from Life Technologies (Burlington, ON). IRDye®800- and 680-conjugated secondary antibodies were purchased from LI-COR Biosciences (Lincoln, NE). Site 1 phosphomutants of rabbit pOSBP (Mohammadi et al., 2001) were further mutagenized to produce pOSBP-S5E (S$_{381,384,387,388,391}$E) and pOSBP-TS6E (T$_{379}$E, S$_{381,387,387,388,391}$E). Site 2 pOSBP-S$_{192,195,200}$D (S3D) and pOSBP-S$_{192,195,200}$A (S3A) were prepared by mutagenesis of pOSBP. Site 1 and Site 2 double mutants were prepared by restriction digestion and ligation of DNA fragments containing mutated sites. All constructs were verified by sequencing and contained three silent mutations that prevented silencing in CHO cells expressing shOSBP.

Cell culture and transfection

CHO cells were cultured in DMEM with 5% fetal calf serum (Medium A). Stable silencing of OSBP in CHO cells was achieved by stable expression of a lentiviral
shOSBP (Banerji et al., 2010). Plasmids encoding OSBPs were transfected into CHO cells using Lipofectamine 2000 according to manufacturers instructions. SF21 insect cells were cultured in SF900-II medium containing 5% (v/v) FBS, 10 µg/mL G418 and 0.25 µg/ml fungizone in monolayer or suspension at 27°C.

**Baculovirus expression and purification of OSBP**

The cDNAs for OSBP, OSBP-S381A, OSBP-S3E, OSBP-RR_{109,110}EE (RR/EE) and OSBP-S5E were cloned into pENTR/D-Topo, verified by sequencing, inserted into linearized BaculoDirect (C-terminal V5-His tagged) by recombination, and transduced into Sf21 cells until a titre of 1x10^8 pfu/ml was achieved. Sf21 cells were infected at an MOI of 0.1 for 1 h at 20°C and resuspended in Sf-900 II media. After culturing in suspension for 72 h, cells were lysed, and His-tagged OSBPs were purified by a 2-step protocol (Ngo and Ridgway, 2009). Following the last step, OSBPs were concentrated to 1-1.5 mg protein/ml in Tris-HCl (pH 7.4) and 250 mM NaCl using a 30 kDa cut-off centrifugal filter concentrator (Millipore) and stored at -80°C.

**Sterol binding, extraction and transfer assays**

The specific binding of 25OH by purified OSBP and OSBP mutants (12 pmol protein) was carried out in 75 µl of 10 mM HEPES (pH 7.4), 100 mM KCl, 2% (w/v) polyvinyl alcohol and [³H]25OH at 4°C for 16 h. A charcoal-dextran slurry (45 µl) was added for 30 min, sedimented by centrifugation at 10,000xg for 10 min, and protein-bound sterol in the supernatant was measured by liquid scintillation counting. Specific binding is defined as total [³H]25OH binding minus binding in the presence of a 40-fold excess of unlabelled 25OH.
[\textsuperscript{3}H]Cholesterol binding assays were similar as described above expect that 0.05% Triton X-100 was included. After incubation at 20°C for 2 h, OSBP-bound [\textsuperscript{3}H]cholesterol was isolated by incubation with 12 μl of Talon affinity resin for 30 min with constant mixing. The resin was washed four times with 500 μl of 10 mM HEPES/100 mM KCl and collected by centrifugation at 5,000xg for 1 min. OSBP-bound [\textsuperscript{3}H]cholesterol was released from the resin with 150 mM imidazole and quantified by liquid scintillation counting.

The liposomal [\textsuperscript{3}H]cholesterol extraction assay was previously described (Ngo and Ridgway, 2009). The cholesterol transfer activity of OSBP and phospho-mutants was assayed by a modified method (Ngo and Ridgway, 2009). Donor and acceptor liposomes composed of [\textsuperscript{14}C]PC:PE:PS:[\textsuperscript{3}H]cholesterol (69:20:10:1 mol/mol) and PC:PE:PS:PIPs:lactosyl-PE (58:20:10:2:10 mol/mol), respectively, were prepared by extrusion and sedimented at 15,000xg for 5 min prior to use. [\textsuperscript{14}C]PC was included in donor liposomes to correct for contamination of acceptor liposomes after sedimentation. The assay was modified to include a pre-extraction step prior to addition of acceptor liposomes. Donor liposomes (10 nmol) were incubated with 100 pmol of recombinant OSBPs and 3 μg of fatty acid-free BSA in 80 μl of liposome buffer (25 mM HEPES, 150 mM NaCl and 1 mM EDTA, pH 7.4) at 25 °C. After 20 min, assays receive 1) 20 μL of liposome buffer and were placed on ice (to measure extraction) or 2) 20 μl of acceptor liposomes (10 nmol) containing 2 mol% PI or PI(4)P for 10 min at 25°C before stopping the reaction on ice (to measure transfer). Acceptor liposomes were precipitated with 10 μg of R. communis agglutinin at 15,000xg for 5 min, and radioactivity in the supernatant and pellet was measured by liquid scintillation counting. Extraction and transfer values
were expressed as a percentage of total $[^3\text{H}]$cholesterol input corrected for donor liposome contamination and transfer in the absence of OSBP.

**PI(4)P competition and extraction assays**

$[^{32}\text{P}]$PI(4)P was isolated from HeLa cells incubated for 24 h in phosphate-free media containing 0.5 mCi/ml of $^{32}\text{PO}_4$. Cells were harvested in 1 ml of PBS, followed by addition of 4 ml of CHCl$_3$/MeOH/12N HCl (2:4:0.1, v/v), 1.2 ml of CHCl$_3$ and 1.2 ml water. The organic phase was dried under nitrogen, resolved by thin-layer chromatography in CHCl$_3$/MeOH/4M NH$_4$OH (90:70:20, v/v) and $[^{32}\text{P}]$PI(4)P identified by autoradiography and co-migration with an authentic standard (purification was repeated once more). Competitive extraction assays were performed using liposomes containing 1 mol% $[^3\text{H}]$cholesterol and increasing mol% of PI, PI(4)P or PI(4,5)P$_2$. Direct extraction of 0.5% $[^3\text{H}]$PI or $[^{32}\text{P}]$PI(4)P by OSBP from cholesterol-free liposomes was assayed as described for $[^3\text{H}]$cholesterol.

**Immunoblotting and immunoprecipitation**

Total cell lysates were prepared in SDS-PAGE sample buffer (12.5% SDS, 30 mM Tris-HCl, 12.5% glycerol and 0.01% bromophenol blue, pH 6.8), heated at 90°C for 5 min, resolved on SDS-PAGE and transferred to nitrocellulose. Proteins were visualized by incubation with primary antibodies for 1-2 h at 20°C followed by secondary IRDye800- or 680-conjugated antibodies for infrared detection using an Odyssey Infrared Imaging System and quantification using Odyssey Application Software v3.0.

Co-immunoprecipitation of VAP-A and OSBP was carried out as described (Wyles et al., 2002). Briefly, CHO cells expressing OSBP phosphorylation mutants were lysed in PBS containing 5 mM KCl, 2 mM EDTA, 2 mM EGTA, 0.5% Triton X-100 and
pre-cleared by centrifugation at 14,000 xg for 15 min at 4°C. VAP-A was immunoprecipitated from supernatants using a polyclonal antibody and protein A-Sepharose, washed three times with lysis buffer and resuspended in SDS-PAGE sample buffer. Immunoprecipitates were resolved by SDS-8% PAGE and immunoblotted for OSBP using monoclonal 11H9. The degree of VAP-A-OSBP co-immunoprecipitation was quantified relative to OSBP input.

**Immunofluorescence and transmission electron microscopy**

CHO cells cultured on glass coverslips were fixed with 4% (w/v) paraformaldehyde in PBS for 15 min and permeabilized with 0.5% (w/v) Triton X-100 for 20 min at 4°C. Coverslips were incubated sequentially with a primary antibody in PBS containing 1% (w/v) BSA at 20°C for 1 h followed by an AlexaFluor-conjugated secondary antibody for an additional 1 h at 20°C. Coverslips were mounted on glass slides with Mowiol® 4-88 and images were captured using a Zeiss LSM510/AxioVert 200M inverted microscope with plan-apochromat 100x/1.40 NA oil immersion objective or a Zeiss LSM510 laser scanning confocal upright microscope with a plan-apochromat 63x/1.40 NA oil immersion objective.

Thin-section (80-100 nm) TEM of CHO cells was performed as described previously (Lagace and Ridgway, 2005). For immuno-electron microscopy, CHO-K1 cells were fixed with 4% (w/v) paraformaldehyde/0.5% (v/v) glutaraldehyde in 0.1M sodium cacodylate buffer for 30 min. Thin-sections mounted on nickel grids were incubated with OSBP monoclonal 11H9 overnight at 4°C followed by an 1 h incubation with 5 nm colloidal gold-conjugated goat anti-mouse secondary antibody at 20°C. Grids were fixed with 2.5% glutaraldehyde and stained with 2% (w/v) uranyl acetate and lead
citrate. Images were captured using a JEOL JEM 1230 transmission electron microscope at 80 kV and Hamamatsu ORCA-HR digital camera.

**Analysis of PIP binding by OSBP phospho-mutants**

Liposomes composed of PC, PE and containing either PI, PI(4)P or PI(4,5)P$_2$ (70:20:10, mol/mol) were incubated with 100 pmol of purified protein for 25 min at 25°C. Samples were centrifuged at 100,000xg for 30 min, and the supernatant and pellet fractions were analyzed by SDS-PAGE, stained with Coomassie and OSBP distribution was quantified using a LI-COR Odyssey IR imaging system.

Lipids (100-300 pmol) and solvent control were spotted onto a Hybond-C nitrocellulose membrane, which was incubated in blocking buffer (20 mM Tris HCl, pH 7.4, 150 mM NaCl, 3% (w/v) fatty acid-free BSA and 0.1% (v/v) Tween-20) at 4 °C with 50 nM OSBPs for 1 h. Bound OSBPs were visualized with 11H9 monoclonal and IRDye800-conjugated antibodies for infrared detection using an Odyssey Infrared Imaging System.

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**REFERENCES**


Figure 1. OSBP phosphorylation sites and mutations. Site 1, Site 2 and protein kinase D phosphorylation sites and mutations reported in this study are indicated in bold. Abbreviations; PHD, PH domain; SBD, sterol binding domain.
Figure 2. Identification of an OSBP Site 1 phosho-mutant with increased sterol binding activity. (A) Purified OSBP, OSBP-S381A, OSBP-S3E and OSBP-S5E (2 μg each) were resolved by SDS-6% PAGE and stained with Coomassie Blue. (B and C) Specific binding of [3H]25OH and [3H]cholesterol by OSBP (■), OSBP-S381A (▲), OSBP-S3E
(●) or OSBP-S5E (♦) (12 pmol) was assayed as described in Materials and Methods.

(D) Following binding of 100 nM [³H]25OH, OSBP and OSBP-S5E were isolated on Talon resin and resuspended in binding buffer containing 100 nM 25OH at 20°C. At the indicated times, OSBP-bound 25OH was removed by centrifugation and radioactivity in the supernatant was quantified. (E) Increasing amounts of OSBPs (see panel B and C for legend) were incubated with liposomes containing 2 mol% [³H]cholesterol and the extraction of radiolabel into the supernatant was measured. (F) Transfer of [³H]cholesterol between liposomes by OSBP, OSBP-S5E, OSBP-S381A or OSBP-RR/EE was determined using a modified assay that involved pre-extraction of sterols from donor liposomes prior to addition of acceptor liposomes containing 2 mol% PI or PI(4)P. Results in all panels are the mean and SEM of 3 or more experiments using 2-3 different protein preparations.
Figure 3. PI(4)P binding by OSBP is competitive with cholesterol but unaffected by Site 1 phosphorylation. (A) OSBP (50 pmol) was incubated with liposomes containing [³H]cholesterol (1 mol%) and increasing amounts PI, PI(4,5)P₂ or PI(4)P (0 to 2 mol%). The extraction of [³H]cholesterol from liposomes by OSBP into the supernatant is expressed relative to activity in the absence of PIPs. Results are from a representative experiment. (B) The association of OSBP with liposomes during the extraction assay shown in panel A was determined by SDS-PAGE of supernatant (S) and pellet (P) fractions. (C) OSBP (50 pmol) was incubated with liposomes containing 0.5 mol% of [³H]PI or [³²P]PI(4)P and extraction of radioactivity into the supernatant was measured after precipitation of liposomes. Results are the mean and SEM of 3 experiments. (D) Increasing amounts of OSBP (■), OSBP-S381A (▲) or OSBP-S5E (●) were incubated
with liposomes containing 0.5 mol% $[^{32}\text{P}]\text{P}_{4}\text{P}$ and extraction of radioactivity into the supernatant was measured. The results are the mean and SEM of 3 experiments.
Figure 4. PH domain activity and Golgi localization is not affected by OSBP Site 1 mutations. (A) Purified wild-type and OSBP mutants (200 pmol) were incubated with 0, 100, 200 and 500 pmol of PI(4)P, PI(4,5)P$_2$ and PC that was immobilized on nitrocellulose filters. Filters were probed with an OSBP polyclonal and goat anti-rabbit IRDye® 800-conjugated secondary antibodies. (B) The association of OSBP and phospho-mutants with liposomes containing 10 mol% PI, PI(4)P or PI(4,5)P$_2$ was
determined by quantification of distribution in the supernatant (light bars) and pellet (black bars) fractions after centrifugation. Results are from representative experiment. (C) The indicated mCherry-OSBP fusions were transiently expressed for 24 h in OSBP-deficient CHO cells followed by incubation with 25OH (6 μM) or solvent control (NA, no addition) for 2 h and immunostaining with a giantin antibody and goat anti-rabbit AlexFluor-488. (D) The indicated amounts of recombinant OSBP (■), OSBP-S381A (▲) or OSBP-S5E (●) were incubated with GST-VAP-A (50 pmol), complexes were isolated by binding to glutathione-Sepharose, and bound OSBP was quantified by SDS-PAGE and Coomassie staining. Results are the mean and SEM of 3 experiments.
Figure 5. OSBP Site 1 phospho-mutants restore 25OH-activated SM synthesis in OSBP-depleted CHO cells. (A and B) CHO cells deficient in endogenous OSBP were transiently transfected for 48 h with wild-type OSBP and Site 1 phospho-mutants. SM (panel A) and ceramide (panel B) synthesis was measured by [$^3$H]serine incorporation after treatment with 25OH (6 μM, black bars) or solvent control (empty bars) for 6 h. Results are the mean and SEM of 3 experiments. (C) Total cell lysates from mock and
OSBP-transfected cells were resolved by SDS-6%PAGE and immunoblotted with an OSBP polyclonal antibody.
**Figure 6.** A Site 2 phosphorylation motif is adjacent to the PH domain. (A) OSBP and the indicated Site1 and Site 2 mutants were transiently transfected into CHO cells for 48 h. Cells were then harvested for immunoblotting or labeled with $^{32}\text{PO}_4$ (0.3 mCi/ml) for 4 h. $^{32}\text{PO}_4$-labeled OSBPs were immunoprecipitated with monoclonal 11H9, resolved by SDS-PAGE and subject to autoradiography for 12 h at -80°C. (B) $^{32}\text{PO}_4$-labeled OSBPs were digested with trypsin and resolved by 2-dimensional thin-layer electrophoresis and chromatography on cellulose-coated plates (Mohammadi et al., 2001). Arrows indicate the position of the Site 2 phosphopeptide. Asterisks indicate the positions of Site 1 phosphopeptides.
Figure 7. Identification of a Site 1/Site 2 double mutant that does not restore sterol-activation of SM synthesis.  (A) OSBP Site 2 mutants (S3A and S3D) were transiently expressed in OSBP-depleted CHO cells and SM and ceramide synthesis was measured by $[^{3}H]$serine incorporation as described in the legend to Figure 5.  (B) A series of four OSBP Site1/Site2 double phospho-mutants were transiently expressed in OSBP-depleted
CHO cells and SM and ceramide synthesis was measured as described above. The results shown in Panels A and B are the means and SEM of 3 experiments. (C and D) Immunoblot analysis of Site 2 (Panel C) and Site 1/Site 2 double phospho-mutants (panel D) expressed in OSBP-depleted CHO cells.
Figure 8. OSBP S381A/S3D collapses and aggregates the ER. (A) OSBP-depleted CHO cells transiently expressing OSBP-S381A/S3D for 24 h were treated with 25OH (6 μg/ml) or solvent control (no addition, NA) for 2 h. Cells were immunostained with OSBP monoclonal 11H9 and AlexaFluor-594-conjugated antibodies, followed by VAP-A polyclonal and AlexaFluor-488-conjugated secondary antibodies. (B) Following 25OH treatment, CHO cells were immunostained with an OSBP polyclonal and AlexaFluor-594-conjugated antibodies, followed by PI4K IIIβ monoclonal and AlexaFluor-488-conjugated secondary antibodies. Arrows indicate dispersed Golgi staining in expressing cells. (C) CHO cells expressing OSBP-S381A/S3D/FF-AA, treated with or without 25OH, were co-immunostained for VAP-A or giantin as described in panel A. CHO cells expressing OSBP (panel D) or OSBP-S381A/S3D (panel E, F, G) were processed for TEM as described in Materials and Methods. The ER is indicted by arrows; bar, 500 nm. Panel F is a high magnification of the boxed area in panel E. (H and I) OSBP-S381A/S3D was visualized in CHO cells with 11H9 and a goat anti-mouse secondary conjugated to 5 nm colloidal gold particles; bar, 100 nm.
**Figure 9.** Enhanced interaction between VAP-A and OSBP-S381A/S3D. (A) Triton X-100 extracts were prepared from OSBP-depleted CHO cells transiently expressing the indicated single OSBP and Site 1 or Site 2 mutants and treated without (-) or with (+) 25OH (6 μM for 2 h). VAP-A was immunoprecipitated from extracts with a polyclonal antibody and immunoblotted for OSBP and VAP-A. (B) OSBP was co-immunoprecipitated with VAP-A from Triton X-100 extracts of OSBP-depleted CHO cells expressing Site 1/Site 2 double phosphomutants. (C) The amount of OSBP co-immunoprecipitated with VAP-A in panel B was quantified as a percentage of total OSBP input (average and range of 2 experiments).