Regulation of Oxysterol-binding Protein Golgi Localization through Protein Kinase D–mediated Phosphorylation

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Submitted February 3, 2010; Revised April 9, 2010; Accepted April 22, 2010

Protein kinase D (PKD) plays a critical role at the trans-Golgi network by regulating the fission of transport carriers destined for the plasma membrane. Two known Golgi-localized PKD substrates, PI4-kinase IIIβ and the ceramide transfer protein CERT, mediate PKD signaling to influence vesicle trafficking to the plasma membrane and sphingomyelin synthesis, respectively. PKD is recruited and activated at the Golgi through interaction with diacylglycerol, a pool of which is generated as a by-product of sphingomyelin synthesis from ceramide. Here we identify a novel substrate of PKD at the Golgi, the oxysterol-binding protein OSBP. Using a substrate-directed phospho-specific antibody that recognizes the optimal PKD consensus motif, we show that PKD phosphorylates OSBP at Ser240 in vitro and in cells. We further show that OSBP phosphorylation occurs at the Golgi. Phosphorylation of OSBP by PKD does not modulate dimerization, sterol binding, or affinity for PI(4)P. Instead, phosphorylation attenuates OSBP Golgi localization in response to 25-hydroxycholesterol and cholesterol depletion, impairs CERT Golgi localization, and promotes Golgi fragmentation.

INTRODUCTION

The Golgi apparatus controls secretory traffic through generation of vesicles that transport lipid and protein cargo destined for the plasma membrane (Warren and Malhotra, 1998; Emr et al., 2009). Despite the constant flux of lipids such as cholesterol, ceramide, and phosphoinositides, the Golgi maintains a unique lipid composition. Several lipid-modifying and -binding proteins function as regulators of Golgi lipid homeostasis. The Golgi is enriched in phosphatidylinositol 4-phosphate [PI(4)P], achieved by the phosphorylation of phosphatidylinositol by PI4-kinase IIIβ (PI4KIIIβ; Haussner et al., 2005, 2006; Weixel et al., 2005). Nir2, a PI-phosphatidylcholine transfer protein, is essential for Golgi vesicle transport through regulation of diacylglycerol (DAG; Vihtelic et al., 1993; Litvak et al., 2005). In addition, phosphatidylinositol-four-P adaptor proteins bind PI(4)P at the Golgi and regulate the formation of transport vesicles (Godi et al., 2004). Similarly, the ceramide transfer protein (CERT) transports ceramide from the endoplasmic reticulum (ER) to the Golgi complex, a process that is critical for sphingomyelin (SM) synthesis and production of DAG (Perry and Ridgway, 2005). A distinct Golgi-localized protein, the oxysterol-binding protein (OSBP), acts as a global sterol sensor or transfer protein for cholesterol and 25-hydroxycholesterol (25-OH). Importantly, both PI4KIIIβ and CERT are substrates of protein kinase D (PKD; Haussner et al., 2005; Fugmann et al., 2007), a serine/threonine kinase that also localizes to the Golgi (Van Lint et al., 2002).

PKD comprises a family of protein kinases in the calcium–calmodulin kinase family of the kinome. Three distinct isoforms exist in mammals: PKD1/PKCμ, PKD2, and PKD3/PKCθ. The PKD regulatory domain comprises two cysteine-rich domains (C1a and C1b) that bind DAG and a pleckstrin homology (PH) domain that functions to autoinhibit the kinase (Van Lint et al., 2002; Rykx et al., 2003). The catalytic domain is highly conserved in all PKD orthologues, and a PDZ (postsynaptic-density-95, Drosophila-discs-large, zona-occludens 1) binding motif is found at the carboxy terminus of PKD1 and PKD2, but not PKD3. In addition to Golgi function, pools of PKDs have been observed at the plasma membrane, cytoplasm, mitochondria, and nucleus where they regulate a variety of cellular processes including cellular proliferation and survival, motility, transcriptional regulation, and immune cell responses (Van Lint et al., 2002; Rykx et al., 2003).

Studies on the function of PKD at the Golgi have revealed that localization at the trans-Golgi network (TGN) is mediated through DAG binding conferred by the C1a domain (Maeda et al., 2001; Baron and Malhotra, 2002). At the TGN, PKD is also activated by novel protein kinase C (PKC) isoforms (Diaz Anel and Malhotra, 2005) and control the fission of transport carriers destined for the plasma membrane (Liljedahl et al., 2001; Yeaman et al., 2004). At the Golgi, PKD transduces signals through phosphorylation and acti-
vation of PI4KIIIβ, leading to increased PI(4)P production (Haussner et al., 2005). CERT, a protein that regulates the transport of ceramide from the ER to the Golgi, is also phosphorylated by PKD thereby decreasing its affinity for PI(4)P (Hanada et al., 2003; Toth et al., 2006). In turn this decreases CERT activity and consequently reduces SM synthesis (Fugmann et al., 2007). Because both CERT and PI4KIIIβ are lipid-binding and -modifying proteins that are PKD substrates, we sought to identify additional substrates that might contribute to PKD function at the Golgi. Here we report the identification of a new PI4KIIIβ substrate, OSBP, that is phosphorylated and regulated at the Golgi.

OSBP is a member of the oxysterol-binding protein family that includes 11 other OSBP-related proteins (ORPs). OSBP and ORPs are characterized by a carboxy-terminal sterol-binding domain that binds sterols such as 25-OH and cholesterol (Lehto and Olkkonen, 2003). OSBP localizes to the cytoplasm or with vesicle-associated membrane protein–associated protein (VAP) in the ER (Wyles et al., 2002; Loe wen et al., 2003; Loewen and Levine, 2005). On binding 25-OH, OSBP translocates to the Golgi (Ridgway et al., 1992; Mohammadi et al., 2001). This translocation also occurs when cholesterol and sphingomyelin are depleted. OSBP localizes to the Golgi by binding PI(4)P through PI(4)P domain interaction (Levine and Munro, 1998; 2002). OSBP regulates the sterol-dependent activation and translocation of CERT; thereby integrating cholesterol and SM metabolism (Perry and Ridgway, 2006). OSBP does not directly regulate cholesterol homeostasis by binding 25-OH, but instead senses or transfers sterols as a mechanism to integrate cellular lipid metabolism (Wang et al., 2005; Ngo and Ridgway, 2009).

Here, we provide evidence that PKD phosphorylates OSBP, thereby inhibiting Golgi localization in response to 25-OH and cholesterol depletion.

MATERIALS AND METHODS

DNA constructs, Reagents, and Antibodies

The anti-PKD (C20) and anti-c-Myc (9E10) antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). The anti-PKD pser744/748 antibody was from Cell Signaling (Danvers, MA); anti-OSBP from Proteintech (Chicago, IL); anti-phosphotyrosine from Santa (St. Louis, MO); anti-TGN46 from AbD Serotec (Raleigh, NC); anti-Giantin from Abcam (Cambridge, MA); anti-GM130 from Novus Biologicals (Littleton, CO); and anti-PI(4)KIβ2 from BD Transduction (San Jose, CA).

The anti-vesicular stomatitis virus glycoprotein (VSVG; 8SF11) antibody was a generous gift from Dr. Doug Lyles (Wake Forest University). The anti-HA antibody was purified in-house from the 12CA5 hybridoma. The anti-PKD anti-PI4KIIIβ mouse antibody was described (Doppler et al., 2005). Lipoprotein-deficient bovine serum and human low-density lipoprotein (LDL) were from Biomedical Technologies (Stoughton, MA). All expression plasmids for PKD1 are based on an amino-terminal HA-tagged PKD1 in pcDNA3 and have been described (Storz et al., 2003) in kinase buffer (25 mM Tris-HCl, pH 7.5, 5 mM β-glycerophosphate, 2 mM DTT, 0.1 mM Na3VO4, and 10 mM MgCl2) in the presence of 250 μM cold ATP for 1 h at 30°C. Samples were resolved by SDS-PAGE, blotted onto membrane, and detected with the indicated antibodies.

Immunoblotting and Immunoprecipitation

Cells were serum-starved for 24 h and stimulated and/or harvested 48 h after transfection. The cells were lysed in lysis buffer (50 mM Tris/HCl, pH 7.5, 0.5% NP-40, 150 mM NaCl, 1 mM EDTA, pH 7.5, 1 mM Na3VO4, 20 mM NaF, 1 mM DTT, 50 mM calcinucin, 1 mM PMSF) plus protease inhibitor cocktail (Sigma). Either the lysates were used for immunoblot analysis, or proteins were immunoprecipitated with a 2- or 4-h incubation with 1 μg anti-myc or anti-OSBP, respectively, followed by a 2-h incubation with protein G agarose (Amersham Biosciences, Piscataway, NJ). Immune complexes were washed three times with NETN (20 mM Tris/HCl, pH 8.0, 100 mM NaCl, 0.5% NP-40, 1 mM EDTA, pH 8.0) and resolved by SDS-PAGE or were subjected to kinase assays.

In Vitro Kinase Assay

Substrates were expressed in HEK293T cells and immunoprecipitated with myc-tagged PKD1. Immune complexes were incubated with purified hemagglutinin (HA)-tagged PKD1 from baculovirus-infected Sf9 insect cells (Storz et al., 2003) in kinase buffer (25 mM Tris-HCl, pH 7.5, 5 mM β-glycerophosphate, 2 mM DTT, 0.1 mM Na3VO4, and 10 mM MgCl2) in the presence of 250 μM cold ATP for 1 h at 30°C. Samples were resolved by SDS-PAGE, blotted onto membrane, and detected with the indicated antibodies.

Immunofluorescence Microscopy

Cells were fixed twice with PBS and then fixed in PBS containing 2% paraformaldehyde for 10 min. Cells were permeabilized with 0.5% Triton X-100 for 1 min and blocked with 1% BSA for 20 min. Fixing and permeabilizing solutions were made in buffer containing 100 mM KCl, 100 mM HEPES, pH 6.8, 200 mM sucrose, and 1 mM MgCl2. Blocking solution was made in buffer containing 20 mM Tris-HCl, pH 7.5 and 150 mM NaCl. Cells were then incubated with primary antibody diluted in blocking buffer for 1 h followed by three washes in PBS and incubation with secondary antibodies diluted in blocking buffer for 1 h. After washing three times with PBS, coverslips were mounted in ProLong Gold (Invitrogen) and analyzed on a fluorescence microscope (Eclipse TE2000, Nikon, Melville, NY) using a PL Fluor 100×/1.30 NA oil objective lens. Images were processed with Photoshop (Adobe Systems, San Jose, CA).

VSVG Transport Assay

After lentiviral infection, cells were transfected with myc-tagged OSBP plasmids and cultured at 37°C. After 24 h, cells were transfected with bsOS5-VSVG-green fluorescent protein (GFP) and cultured at 40°C for 20 h. Cycloheximide, at 100 μg/ml, was added before a 15-min incubation at 32°C. This was followed by a 2-h incubation at 20°C and a final incubation at 32°C for the indicated times. Cells were fixed with 2% formaldehyde and blocked as described above. Surface VSVG was labeled for 1 h with anti-VSVG mAb 8SF11, which is specific for the extracellular domain of VSVG. After washings with PBS, cells were incubated with secondary Cy3-conjugated anti-mouse IgG for 1 h.

Mass Spectrometry

Co-immunoprecipitated SDS-PAGE gel bands containing myc-OSBP isolated from HEK293T cells with different treatments were excised and subjected to reduction with DTT and alkylation with iodoacetamide in-gel digestion with trypsin, and massings with PBS, cells were incubated with secondary Cy3-conjugated anti-mouse IgG for 1 h. After washing three times with PBS, coverslips were mounted in ProLong Gold (Invitrogen) and analyzed on a fluorescence microscope (Eclipse TE2000, Nikon, Melville, NY) using a PL Fluor 100×/1.30 NA oil objective lens. Images were processed with Photoshop (Adobe Systems, San Jose, CA).
PKD Regulates OSBP Golgi Localization

RESULTS

OSBP Reactivity with PKD pMOTIF Requires PKD Activity

We recently developed a novel PKD phosphorylation-state specific substrate–directed antibody that recognizes the optimal phosphorylation motif of PKD, LXR(Q/K/E/M)(M/L/K/E/Q)/A)S*XXXX (where X denotes any amino acid; Doppler et al., 2005). The PKD pMOTIF antibody has been used to identify several PKD substrates including two novel Golgi-localized PKD targets, P4KIIIB (Hausser et al., 2005) and CERT (Fugmann et al., 2007). We reasoned that addi-
tional Golgi-localized lipid-binding proteins that are also PKD substrates must exist, and screened phosphopeptides recently were identified by functional phospho-proteomics (Villen et al., 2007). Using this approach, we identified a phosphopeptide in human OSBP that conforms to the optimal PKD consensus motif at Ser240 (Figure 1A).

To evaluate whether OSBP is a PKD substrate, we first determined whether OSBP is recognized by the pMOTIF antibody. Cells transfected with Myc-tagged OSBP were stimulated with PdBu, a potent PKD activator, and analyzed by immunoblotting. OSBP immunoreactivity with PKD pMOTIF coincides with PKD phosphorylation at Ser738/Ser742, a surrogate for PKD activation (Figure 1B). Similar kinetics of phosphorylation are also observed with platelet-derived growth factor (PDGF), a physiological agonist of PKD (Figure 1C). Immunoreactivity of OSBP with pMOTIF antibody is dependent on PKD catalytic activity because a constitutively active PKD allele (PKD.SS/EE) induces OSBP phosphorylation, whereas a kinase-inactive allele (PKD.KW) does not (Figure 1D). Moreover, immunoreactivity is completely blocked in cells pretreated with Gö6976, a potent PKD and PKC inhibitor, compared with control PdBu-stimulated cells (Figure 1E). Taken together, these data show that PKD kinase activity is required for OSBP recognition by the pMOTIF antibody.

**PKD Phosphorylates OSBP at Ser242**

The PKD pMOTIF antibody recognizes the phosphorylated consensus motif of other protein kinases such as AMP-activated kinase (AMPK), mitogen-activated protein kinase–activated protein kinase 2 (MAPKAP-K2 or MK2), and calcium–calmodulin-dependent kinases (CamK). To determine whether PKD is the relevant kinase for OSBP phosphorylation, PKD short hairpin RNA (shRNA) was used. PdBu stimulation increases endogenous OSBP phosphorylation as predicted; however, silencing PKD1 expression using lentiviral shRNA reduces OSBP recognition by pMOTIF (Figure 2A). To corroborate these results, a rescue experiment was performed in which OSBP was expressed with constitutively active PKD.SS/EE or an shRNA resistant PKD.SS/EE* allele in cells depleted of PKD1 and PKD2. In the presence of PKD, OSBP is phosphorylated by PKD.SS/EE and PKD.SS/EE* to similar levels (Figure 2B, lanes 3 and 4). Silencing of PKD.SS/EE by PKD1/2 shRNA decreases OSBP phosphorylation (Figure 2B, lane 7). PKD.SS/EE* is refractory to silencing by PKD1/2 shRNA, and this effectively rescues OSBP phosphorylation (Figure 2B, lane 8). Finally, silencing of PKD1 or PKD2 alone or in combination decreases OSBP phosphorylation to similar levels, indicating a lack of isoform specificity in OSBP phosphorylation (Figure 2C). This is in agreement with other Golgi-localized PKD substrates such as PI4KIIIβ and CERT that are also efficiently phosphorylated by PKD1 or PKD2 (Hausser et al., 2005; Fugmann et al., 2007).

Functional phospho-proteomics have revealed OSBP phosphorylation in vivo at Ser240 that conforms to a PKD consensus phosphorylation motif (Villen et al., 2007). This motif is evolutionarily conserved in all mammals and to immunoblotted with anti-HA. (C) HEK293T cells were lentivirally infected with PKD1, PKD2, or PKD1 and PKD2 shRNAs and then transected with myc-OSBP. Cells were serum-starved and stimulated with 1 μM PdBu for 30 min. OSBP was immunoprecipitated with anti-myc and immunoblotted with anti-pMOTIF and anti-myc. Total lysates were immunoblotted with anti-HA.
Danio rerio. Examination of the amino acid sequence of OSBP reveals an additional putative PKD motif corresponding to human Ser269 that is conserved to Drosophila melanogaster (Figure 1A). Both sites conform to a minimal PKD consensus motif with a leucine at −5 and an arginine at −3 relative to the phosphoacceptor (Doppler et al., 2005). A rabbit (Oryctolagus cuniculus) OSBP cDNA was used in these studies, whereby the corresponding PKD phosphorylation sites are Ser242 and Ser271 (Figure 1A). To assess the relative contribution of these sites to OSBP phosphorylation by PKD, wild-type, and Ser-to-Ala OSBP alleles were coexpressed with constitutively active PKD (PKD.SS/EE) and immunoblotted with the pMOTIF antibody. Although wild-type OSBP is efficiently phosphorylated by activated PKD, phosphorylation of OSBP.S242A is completely eliminated (Figure 3A). In contrast, OSBP.S271A phosphorylation is unchanged from wild-type OSBP. Similarly, both wild-type and S271A OSBP are directly phosphorylated by purified PKD in an in vitro protein kinase assay, compared with mutant S242A (Figure 3B). These data demonstrate that PKD directly phosphorlates OSBP at Ser242. To corroborate these data and test for Ser242 and Ser271 phosphorylation using a distinct physiological approach, LC-MS/MS was used. Cells infected with shRNA control or PKD1/PKD2 shRNA were cotransfected with tagged OSBP and constitutively active PKD (SS/EE PKD) or OSBP and SS/EE PKD with PKD1/2 shRNA is relative to cells expressing only OSBP. (E) Expression of OSBP and knockdown of SS/EE PKD were confirmed by immunoblotting with anti-myc and anti-HA. Results are representative of at least three independent experiments.
PKD Phosphorylates OSBP at the Golgi

We next determined if OSBP phosphorylation by PKD occurs at the Golgi, because a pool of PKD localizes at the Golgi where it phosphorylates PI4KIIIβ and stimulates PI(4)P production, and in turn bind OSBP through its PH domain. An OSBP PI(4)P-binding mutant was generated with mutation of Arg109 and Arg110 to Glu, as these are residues in the PH domain that coordinate binding of the phosphate group of PI(4)P. OSBP was depleted from cells using shRNA and either wild-type OSBP or PI(4)P-binding mutant (OSBP.RR109/110EE) that are refractory to silencing were reintroduced. Although wild-type OSBP translocates from a perinuclear compartment to the Golgi upon 25-OH stimulation, OSBP.RR109/110EE has a more cytoplasmic staining pattern with minimal localization to the Golgi, with or without 25-OH treatment, as revealed by costaining with the TGN marker TGN46 (Figure 4A). In a parallel experiment, phosphorylation of the PI(4)P-binding mutant was determined in cells expressing constitutively active PKD (PKD.SS/EE) and either wild-type OSBP or OSBP.RR109/110EE. Immunoblotting with PKD pMOTIF reveals significantly reduced OSBP phosphorylation with the PI(4)P-binding mutant compared with wild-type OSBP (Figure 4B). The residual phosphorylation of OSBP.RR109/110EE can be explained by residual localization of the PI(4)P-binding mutant at the TGN (Figure 4A) under these conditions. Overall, these data indicate that PKD phosphorylation of OSBP occurs at the Golgi.

Ser242 Phosphorylation Affects OSBP trans-Golgi Network Localization

We next investigated the functional consequences of OSBP phosphorylation by PKD. We first evaluated OSBP homodimerization, as OSBP has been shown to exist as homodimers and Ser242 resides in a leucine zipper (Ridgway et al., 1992). Phospho-mimetic OSBP.S242D and OSBP.S242A were used in a glutaraldehyde cross-linking experiment, revealing that neither OSBP.S242D nor OSBP.S242A affect OSBP homodimerization compared with control (Supplemental Figure S1). This is consistent with deletion of the leucine zipper having a negligible effect on OSBP homodimerization (Ridgway et al., 1992). We next determined the effect of OSBP phosphorylation on sterol binding. In vitro sterol-binding assays revealed no significant effects on the maximal binding capacity of OSBP.S242A or OSBP.S242D for 25-OH (Supplemental Figure S2). This is consistent with the distal location of Ser242 from the sterol-binding domain of OSBP. We also evaluated the consequence of OSBP phosphorylation on PI(4)P binding, because OSBP localizes to the Golgi through binding of PI(4)P through its PH domain. However, in vitro lipid-binding assays revealed that neither OSBP.S242A or OSBP.S242D are impaired in PI(4)P binding compared with wild-type OSBP (Supplemental Figure S3).

We next focused on regulation of OSBP Golgi localization in response to PKD signaling. OSBP translocation to the Golgi was examined using indirect immunofluorescence microscopy in cells depleted of endogenous OSBP and expressing wild-type OSBP or mutant OSBP.S242A or OSBP.S242D that are refractory to silencing. To assess Golgi localization, the Golgi markers GM130, Giantin, and TGN46 were used to mark the cis-, cis-/medial-, and trans-Golgi, respectively. 25-OH treatment stimulates translocation of OSBP and OSBP.S242A to the Golgi, evident by colocalization with GM130, Giantin, and TGN46 (Figure 5, A–C). Similarly, 25-OH stimulates translocation of OSBP.S242D to the cis- and medial-Golgi (Figure 5, A and B). Moreover, under these conditions there is a minor pool of OSBP that does not colocalize with any one Golgi marker, consistent with the notion that OSBP is a medial- and trans-Golgi–associated protein. In contrast, OSBP.S242D does not localize to the TGN upon 25-OH stimulation, with OSBP and TGN46 staining distinct cellular compartments with no detectable colocalization (Figure 5C). These data indicate that phosphorylation of Ser242 specifically inhibits 25-OH-dependent OSBP localization to the TGN, whereas OSBP localization to the cis- and medial Golgi is not altered. This is consistent with PKD activity at the TGN and its established role in regulating the fission of budding vesicles (Liljedahl et al., 2001). To further investigate this functional mechanism of OSBP localization, we evaluated localization in response to cholesterol depletion. OSBP translocates to the Golgi in response to altered cholesterol trafficking and depletion of plasma membrane cholesterol (Ridgway et al., 1992; Storey et al., 1998). Experimentally, OSBP constitutively lo-
calizes to the Golgi in response to cholesterol depletion by treatment with cyclodextrin (Ridgway et al., 1992) or treatment with delipidated serum (Ridgway et al., 1992; Storey et al., 1998). Repletion of cellular cholesterol with LDL leads to the dissociation of OSBP from the Golgi (Storey et al., 1998). Cells depleted of endogenous OSBP with shRNA and reexpressing wild-type OSBP, OSBP.S242A, or OSBP.S242D that are refractory to silencing were grown in lipoprotein-deficient serum (LPDS) for 24 h and then stimulated with LDL. As previously reported, OSBP staining revealed constitutive localization to a concentrated perinuclear region that colocalizes with TGN46 and previously confirmed as the Golgi (Mohammadi et al., 2001; Figure 6A, WT.OSBP, S242A, or S242D). Cells were grown in 5% lipoprotein-deficient serum (LPDS) for 24 h and treated with 50 μg human LDL/ml for 18 h. Cells were then fixed and immunostained with anti-myc or anti-TGN46. Results are representative of at least three independent experiments. (B) Quantitation of Golgi fragmentation was determined by counting the number of cells with dispersed TGN46 staining (to total number of cells with fragmented and nonfragmented Golgi). The percentage of cells with Golgi fragmentation was determined for OSBP-depleted cells expressing the indicated mutants −/−LDL. Statistical evaluations were made between the following: WT and S242A, +LDL; WT and S242D, −LDL; and WT and S242D, +LDL. The data shown are mean values ± SD of four independent experiments, with n = 100 for each condition. *p < 0.05; **p < 0.01; ***p < 0.001.

Constitutive Ser242 Phosphorylation Promotes Golgi Fragmentation

We noticed that under conditions where OSBP displays a diffuse cytoplasmic localization (Figure 6A, WT.OSBP +LDL, S242D −/+LDL), TGN46 staining is dispersed, suggesting that inhibition of OSBP Golgi localization may pro-
mote fragmentation of the Golgi. Quantitation of cells with dispersed TGN46 staining confirms that increased Golgi fragmentation in cells expressing OSBP.S242D compared with wild-type is significant before \((p < 0.001)\) and after \((p < 0.01)\) LDL treatment (Figure 6B). Furthermore, the decrease in fragmentation in cells expressing OSBP.S242A after LDL treatment is also significant \((p < 0.05)\), indicating that constitutive localization of OSBP at the Golgi prevents fragmentation. This is consistent with previous reports that have demonstrated complete vesiculation of the Golgi apparatus upon PKD activation (Bossard et al., 2007). These results indicate that nonfunctional OSBP that is impaired in Golgi localization also promotes fragmentation. This finding may explain the significant inhibition of 25-OH–activated SM synthesis upon cellular depletion of OSBP (Perry and Ridgway, 2006). Consistent with this, Golgi fragmentation is also induced upon depletion of the OSBP family member OSBP-related protein 9 (ORP9L; Ngo and Ridgway, 2009).

Ser242 Phosphorylation Affects CERT Golgi Localization

We next investigated whether alterations in OSBP localization affect CERT localization, as 25-OH–induced OSBP Golgi translocation is required for CERT localization and activation (Perry and Ridgway, 2006). 25-OH treatment stimulates translocation of OSBP and OSBP.S242A from a perinuclear/cytoplasmic compartment to the Golgi, whereas OSBP.S242D fails to translocate (Figure 7). In all conditions, a significant pool of CERT colocalizes with OSBP. As OSBP.S242D fails to localize to the TGN with 25-OH treatment (Figure 5), we conclude that CERT also fails to translocate to the TGN in cells expressing OSBP.S242D. Taken together, these data demonstrate that inhibition of 25-OH–induced OSBP Golgi localization by PKD-mediated Ser242 phosphorylation leads to the impairment of CERT translocation to the Golgi.

OSBP Is Required for TGN-to-Cell Surface Protein Transport

Because PKD is required for secretory transport (Bossard et al., 2007), we next determined whether OSBP is also required for delivery of cargo destined for exocytosis. To investigate this, cells depleted of endogenous OSBP with shRNA were transfected with myc-tagged WT.OSBP, S242A, and S242D plasmids refractory to silencing. Cells were then transfected with the tsO45 strain of VSVG-GFP with a thermosensitive mutation causing it to unfold in the ER at the nonpermissive temperature of 40°C. After 24 h, cells were shifted to 32°C in the presence of cycloheximide to allow protein folding and then to 20°C to accumulate tsO45-VSVG in the Golgi. On shifting cells to the permissive temperature of 32°C, tsO45-VSVG is transported to the cell surface. VSVG transported to the cell surface is detected by immunofluorescence with an exofacial antibody (8G5F11) that recognizes the extracellular portion of VSVG. In control cells, VSVG is transported to the cell surface 60 min after temperature shift, whereas there is a severe delay in OSBP-depleted cells that is most evident at 120 min (Figure 8). The delay is rescued with WT.OSBP and OSBP.S242A, but not with OSBP.S242D. Furthermore, the level of intracellular VSVG-GFP localized to the Golgi decreases over time in cells in which the secretory pathway is intact, but remains in the Golgi when the secretory pathway is disrupted (Supplemental Figure S4). The defect in VSVG transport is likely a result of Golgi fragmentation that occurs upon OSBP depletion (Supplemental Figure S5). These data demonstrate that OSBP is required for proper Golgi structure and trafficking of cargo from the Golgi to the plasma membrane.

**Figure 7.** Ser242 phosphorylation affects CERT Golgi localization. CHO-K1 cells stably expressing CERT-GFP were transiently transfected with myc-WT OSBP, S242A, or S242D. Cells were treated with 2.5 μg/ml 25-OH for 2 h. Cells were fixed and immunostained with anti-myc. Results are representative of at least three independent experiments.

**Figure 8.** Depletion of OSBP impairs VSVG transport. HeLa cells infected with OSBP shRNA were transiently transfected with myc-WT.OSBP, S242A, or S242D. After 24 h, cells were transfected with tsO45-VSVG-GFP. Cells were fixed and immunostained with anti-VSVG (8G5F11) to detect surface VSVG after the indicated times (min). Results are representative of at least three independent experiments.

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PKD phosphorlates OSBP on Ser242 (Figure 3) located in the putative leucine zipper region (Figure 1A). This region fits the criteria for α-helical sequences that mediate dimerization of certain DNA-binding proteins (Landschulz et al., 1988), with the exception that it lacks an adjacent basic domain that binds DNA. Although not required, the leucine zipper does contribute to OSBP homodimerization. Deletion of this domain results in dimerization; however, it is significantly reduced compared with wild-type OSBP, suggesting that the leucine zipper may function to stabilize homodimerization (Ridgway et al., 1992), although it is dispensable for initial dimer formation. Consistent with this, Ser242 mutations in the leucine zipper do not affect OSBP homodimerization (Supplemental Figure 1). This does not preclude the possibility that the leucine zipper facilitates heterodimerization with other proteins. Studies have shown that phosphorylation in the leucine zipper stabilizes multimeric complexes (Loriaux et al., 1993; Szilak et al., 1997b) and also disrupts α-helices (Szilak et al., 1997a). The importance of phosphorylation in regulating leucine zipper-mediated protein–protein interactions suggests that PKD phosphorylation of OSBP may regulate OSBP interaction with other proteins.

PKD phosphorylation of OSBP regulates TGN localization in response to 25-OH treatment or cholesterol depletion (Figures 5C and 6A). As OSBP phosphorylation occurs at the TGN (Figure 4), this localization defect is likely due to the inability of OSBP to associate with the TGN upon phosphorylation. As the affinity for PI(4)P is unaffected in vitro (Supplemental Figure 3), it is likely that other factors at the TGN whose interactions with OSBP, possibly through the leucine zipper domain, are regulated by PKD phosphorylation. Phosphorylation may promote an interaction with a ternary binding protein, which serves to mask the PH domain of OSBP, thereby inhibiting PI(4)P binding. Interaction with a ternary protein may also inhibit ARF1 interaction. In addition to PI(4)P, studies have shown that the ARF1 GTPase is critical for OSBP Golgi localization (Levine and Munro, 2002). At the Golgi, the PH domains of OSBP (OSBP-PH) and FAPP (FAPP-PH) bind activated ARF1 (GTP-ARF1). This facilitates the stabilization of ARF1 at Golgi membranes by interfering with the interaction with ARF-GAP, effectively inhibiting recruitment to the Golgi and reducing GAP.

DISCUSSION

In this study, we identify OSBP as a novel substrate of PKD at the Golgi and provide the first demonstration of phosphorylation-mediated regulation of OSBP function. The mechanism by which PKD regulates Golgi structure and function is defined by its substrates. PKD phosphorylation of PI4KIIIβ stimulates its kinase activity leading to increased PI(4)P production (Hausser et al., 2005, 2006). In addition to maintaining local PI(4)P levels at the TGN, PKD regulates Golgi lipid homeostasis through phosphorylation of another Golgi-localized substrate, CERT. PKD-mediated phosphorylation decreases the affinity of the CERT PH domain for PI(4)P, thereby inhibiting transport of ceramide, a SM precursor, from the ER to the Golgi (Fugmann et al., 2007). The inhibition of OSBP Golgi localization by phosphorylation impairs the 25-OH-induced OSBP localization of CERT (Figure 7). This is consistent with previous studies demonstrating the requirement of 25-OH-induced OSBP Golgi localization for CERT activity and SM synthesis (Perry and Ridgway, 2006). This suggests that PKD-mediated inhibition of OSBP Golgi association may affect CERT activity and SM synthesis, thus acting as a distinct mechanism of PKD signaling that influences SM metabolism. We propose that by phosphorylating OSBP and inhibiting its association with the Golgi, PKD regulates the ability of CERT to localize to the Golgi. In turn, PKD-mediated phosphorylation of CERT causes its release from the Golgi. The net effect is rapid termination of SM synthesis. SM and cholesterol are coregulated metabolically and associate physically in membrane microdomains involved in cargo sorting and signaling. An excess of these lipids would perturb the rigidity of and signaling from endomembranes. In line with this notion, disruption of lipid rafts by cholesterol depletion with methyl-β-cyclodextrin activates PKD (Cabrera-Poch et al., 2004). Termination of SM synthesis through substrate phosphorylation of OSBP and CERT may explain coordinate downregulation of SM synthesis that is observed upon cholesterol depletion.

membrane and that constitutive phosphorylation of OSBP impairs this process.
activity (Godi et al., 2004). Brefeldin A disrupts the Golgi by interfering with the ARF1 and ARF-GEF interaction, resulting in ARF1 inactivation (Cherfils and Melancon, 2005). Consistent with this, we demonstrate that a phospho-mimetic OSBP mutant (OSBP.S242D) is impaired in Golgi localization, and this is concomitant with increased Golgi fragmentation (Figure 6). We also show that OSBP depletion induces Golgi fragmentation (Supplemental Figure S5). It is worth noting that depletion of ORP9L also leads to Golgi fragmentation (Ngo and Ridgway, 2009). In further support of this model, OSBP that is constitutively localized to the Golgi protects from Golgi fragmentation (Ngo and Ridgway, 2009). In this case, the increased Golgi fragmentation observed with OSBP.S242D expression is consistent with a model in which the OSBP that is phosphorylated by PKD at S242 is nonfunctional. This is also reminiscent of the functional role of PKD activation that leads to Golgi vesiculation (Bossard et al., 2007). We also show that OSBP is required for trafficking of cargo from the TGN to the plasma membrane and that constitutive phosphorylation of Ser242 impairs the secretory pathway. As complete vesiculation of Golgi membranes inhibits TGN to plasma membrane transport (Takizawa et al., 1993), our results are consistent with a model in which hyperactivation of PKD leads to constitutive phosphorylation of OSBP and Golgi fragmentation, which in turn impairs transport of cargo.

OSBP also serves as a cholesterol-sensitive scaffolding protein for two extracellular signal-regulated kinase (ERK) phosphatases, PP2A and HePTP, to regulate ERK1/2 activation. Depletion of cellular cholesterol with methyl-β-cyclodextrin triggers disassembly of the oligomer and leads to an increase in the level of pERK1/2. It has been reported that 25-OH treatment leads to increased ERK1/2 activation (Ares et al., 2000). This is most likely due to oligomer disassembly and inhibition of pERK1/2 dephosphorylation, as exposure to 25-OH results in disassembly of the OSBP/PP2A/HePTP oligomer (Wang et al., 2005). As both cholesterol depletion and 25-OH induce OSBP Golgi localization, we speculate that PKD-mediated phosphorylation and inhibition of OSBP Golgi localization also attenuate ERK1/2 activation.

PKD is a critical regulator of Golgi structure and function through substrate phosphorylation of PI4KIIβ, CERT, and OSBP. Our results are consistent with a model in which PKD phosphorylation of PI4KIIβ leads to increased PI(4)P production at the TGN, allowing CERT and OSBP to localize to the Golgi through their PH domains (Figure 9). Phosphorylation of CERT attenuates SM and DAG production (Kumagai et al., 2007). Similarly, phosphorylation of OSBP causes its displacement from the Golgi. As OSBP Golgi localization regulates CERT activity, we propose that PKD phosphorylation of OSBP regulates localization of SM and DAG by steroid sensing or a direct transfer activity that controls sterol levels in the Golgi (Ngo and Ridgway, 2009). Thus, PKD phosphorylation of CERT and OSBP may serve to tightly integrate SM and cholesterol levels at the Golgi, which in turn has a profound effect on lipid raft assembly and maintenance of lipid homeostasis in the cell.

ACKNOWLEDGMENTS

We thank Dr. Richard G. W. Anderson for providing the OSBP cDNA and also thank Drs. Lewis C. Cantley, Andrius Kazlauskas, and John Blenis for guidance, and all members of the Toker laboratory for discussions and advice. This work was supported in part by National Institutes of Health (NIH) Grant CA075134 (A.T.); Canadian Institutes of Health Research Grants MOP-15284 (N.R.), SP01CA120964-03, and SF90CA06656-45S6 (J.A.); the Burroughs Wellcome Fund and NIH Director’s New Innovator Award (S.F.); and the American Cancer Society Postdoctoral Scholar Award (M.N.).

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PKD Regulates OSBP Golgi Localization


