Cholesterol regulates oxysterol binding protein (OSBP) phosphorylation and Golgi localization in Chinese hamster ovary cells: correlation with stimulation of sphingomyelin synthesis by 25-hydroxycholesterol

Margo K. STOREY*†[1], David M. BYERS*†, Harold W. COOK*† and Neale D. RIDGWAY*†[2]

*Department of Pediatrics, Atlantic Research Centre, Dalhousie University, 5849 University Avenue, Halifax, Nova Scotia, Canada, B3H 4H7, and †Department of Biochemistry, Atlantic Research Centre, Dalhousie University, 5849 University Avenue, Halifax, Nova Scotia, Canada, B3H 4H7

Sphingomyelin (SM) and cholesterol content is positively correlated in cellular membranes, and in several pathological and experimental conditions there is evidence for coregulation. The potential role of oxysterols and oxysterol binding protein (OSBP) in mediating the coregulation of cholesterol and SM was examined using Chinese hamster ovary (CHO) and cholesterol auxotrophic, sterol regulatory defective (SRD) 6 cells. SRD 6 cells grown in the presence or absence of cholesterol for 24 h displayed a 30–50 % reduction in SM synthesis compared with control CHO 7 cells. SM synthesis in CHO 7 and cholesterol-supplemented SRD 6 cells was stimulated 2-fold by 25-hydroxycholesterol, but cholesterol-starved SRD 6 cells were unresponsive. Basal and 25-hydroxycholesterol-stimulated SM synthesis was also inhibited in lovastatin-treated wild-type CHO-K1 cells. Lack of 25-hydroxycholesterol activation of SM synthesis in cholesterol-starved SRD 6 and lovastatin-treated CHO-K1 cells was correlated with dephosphorylation of OSBP. In SRD 6 cells, this was evident after 12 h of cholesterol depletion, it occurred equally at all phosphorylation sites and was exacerbated by 25-hydroxycholesterol. Unlike CHO 7 cells, where OSBP was observed in small vesicles and the cytoplasm, OSBP in cholesterol-starved SRD 6 cells was constitutively localized in the Golgi apparatus. Supplementation with non-lipoprotein cholesterol promoted redistribution to vesicles and the cytoplasm. Similarly, OSBP in CHO-K1 cells grown in delipidated serum was predominantly in the Golgi apparatus. Low-density lipoprotein (LDL) supplementation of CHO-K1 cells caused the redistribution of OSBP to the cytoplasm and small vesicles, and this effect was blocked by pharmacological agents {3-β-[2-(diethylamino)ethoxy]androst-5-en-17-one and progesterone}, which inhibited LDL cholesterol efflux from lysosomes. The results showed that localization of OSBP between the Golgi apparatus and a cytoplasmic/vesicular compartment was responsive to changes in cholesterol content and trafficking. In cholesterol depleted SRD 6 cells, this was accompanied by dephosphorylation of OSBP and attenuation of 25-hydroxycholesterol activation of SM synthesis.

INTRODUCTION

The tissue and cell content of cholesterol and sphingomyelin (SM) is reported to change coordinately under various pathological and experimental conditions (reviewed in [1]). This relationship is also seen in subcellular membranes where the relative content of cholesterol and SM are positively correlated [2]. This is particularly true of the plasma membrane which contains 60–80 % of total cholesterol and SM [3,4]. Co-localization of cholesterol and SM in the plasma membrane has important regulatory implications as demonstrated by the activation of cholesterol esterification [5], inhibition of sterol-regulatory-element binding protein (SREBP) processing [6] and cholesterol synthesis [7] in cells treated with exogenous bacterial sphingomyelinase. Association of cholesterol and SM in plasma membrane structures such as caveolae [8] could explain the dependence of cholesterol localization and regulation on plasma membrane SM content. It has been proposed that SM modulates the capacity of the cell to accommodate cholesterol, thus functioning as a set point by dictating when excess cholesterol is available for regulatory purposes and esterification at the endoplasmic reticulum [9].

Co-variance of cholesterol and SM in cells could be a passive process, whereby the absolute level of one lipid dictates the capacity of the membrane to accommodate the other. Alternatively, synthesis or catabolism could be directly affected by sterol or sphingolipid metabolites. There is conflicting evidence for regulation of SM synthesis by cholesterol. Low-density lipoprotein (LDL) is reported to inhibit SM synthesis in fibroblasts [10] and renal tubular cells [11], but was without effect in CHO-K1 cells [12]. Acetyl-LDL loading of macrophages profoundly increased SM content but it was unclear what proportion came from *de novo* synthesis [13]. Drugs that alter cholesterol synthesis, such as 3-β-[-2-(diethylamino)ethoxy]androst-5-en-17-one (U18666A), lovastatin and 25-hydroxycholesterol, did not alter [^{3}H]serine incorporation into long-chain bases of CaCo-2 cells [14]. Similarly, inhibition of SM production by fumonisin [15], β-chloroalanine and cycloserine [14] had negligible effects on cholesterol synthesis. However, altering the cellular content of cholesterol or SM with exogenous supplementation caused independent changes in the biosynthetic rates for each lipid [14,16]. It appears that synthetic, and perhaps degradative, rates respond primarily to alterations in membrane cholesterol/SM ratios and not to acute changes in synthesis of lipid or sterol.

Abbreviations used: ACAT, acyl-CoA:cholesterol acyltransferase; CHO, Chinese hamster ovary; DMEM, Dulbecco's modified Eagle's medium; LDL, low-density lipoprotein; LPDS, lipoprotein-deficient serum; OSBP, oxysterol binding protein; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdSer, phosphatidylserine; SM, sphingomyelin; SREBP, sterol-regulatory-element binding protein; SPT, serine palmitoyltransferase; SRD; sterol regulatory defective; 58035, ACAT inhibitor; U18666A, 3-β-[2-(diethylamino)ethoxy]androst-5-en-17-one; Ab, antibody.

[1] Present address: National Jewish Medical and Research Center, Department of Medicine, 1400 Jackson St., Denver, CO 80206, U.S.A.

[2] To whom correspondence should be addressed (email nridgway@is.dal.ca).

It has been reported previously that 25-hydroxycholesterol could be a regulator of cholesterol and SM synthesis in CHO-K1 cells. 25-Hydroxycholesterol caused changes in SM synthesis that coincided with inhibition of transcription of sterol-regulated genes and activation of cholesterol esterification. These effects included increased SM mass and synthesis, primarily at the level of ceramide conversion to SM [17]. While the role of oxysterols as physiological regulators of cholesterol metabolism has yet to be established, 25-hydroxycholesterol has been shown repeatedly to initiate regulatory responses indicative of an increased cellular cholesterol load when added to cultured cells [18]. The simultaneous increase in SM production seen in CHO-K1 cells may be a mechanism to buffer the cell against the adverse effects of increased cholesterol.

How 25-hydroxycholesterol elicits changes in synthesis of SM and cholesterol is unknown, but transduction via a non-nuclear receptor termed the oxysterol binding protein (OSBP) is a possibility. OSBP is a low-abundance, high-affinity receptor for a variety of oxysterols such as 25-hydroxycholesterol [19], the binding of which promoted translocation of OSBP from a cytoplasmic/vesicular compartment to the Golgi apparatus [20]. When overexpressed in CHO cells, OSBP has primary or secondary effects on cholesterol homoeostasis, indicated by increased expression of sterol-regulated genes, increased cholesterol synthesis and suppression of acyl-CoA:cholesterol acyltransferase (ACAT) activity [21]. In the same overexpressing cells, stimulation of SM synthesis by 25-hydroxycholesterol was 2- to 3-fold greater than in mock transfected controls (T. A. Lagace and N. D. Ridgway, unpublished work). The Golgi apparatus is the site for conversion of ceramide to SM [22], and some pathways of intracellular cholesterol trafficking and regulation have been localized to this organelle [23,24]. Thus OSBP could modulate Golgi-specific activities related to sphingolipid and cholesterol synthesis and trafficking.

In the present study, the effect of cholesterol on SM biosynthesis was examined using cholesterol auxotrophic SRD 6 cells as a model. These cells have a defect in the second, non-sterol-regulated proteolytic cleavage of SREBP-1 and -2, which renders these transcription factors constitutively membrane bound and unable to migrate to the nucleus [25]. Cholesterol synthetic rates in SRD 6 cells are 5–10 % of that of control cells [26], and total cholesterol content is decreased by 40 % when cells are grown in cholesterol-free medium for 24 h [27]. In the present study, we show that cholesterol influenced the capacity of SRD 6 cells to stimulate SM synthesis in response to the OSBP ligand 25-hydroxycholesterol. The lack of stimulation of SM synthesis by 25-hydroxycholesterol was correlated with constitutive Golgi localization and dephosphorylation of OSBP. Wild-type CHO-K1 cells grown in lipoprotein-free medium also displayed Golgi localization of OSBP, and dissociation from the Golgi complex required LDL cholesterol release from the lysosome.

MATERIALS AND METHODS

Materials

Cholesterol complexed with methyl-β-cyclodextrin, methyl-β-cyclodextrin, progesterone and mevalonate were purchased from Sigma Chemical Co. 25-Hydroxycholesterol was purchased from Steraloids. [G-^{3}H]Serine, [9,10-^{3}H]oleate and [^{32}P]P_i were from Mandel Scientific–NEN Life Science Products. Protein A-Sepharose and glutathione-Sepharose were from Pharmacia–LKB. Lovastatin was from Merck Frosst Canada (Kirkland, Quebec, Canada). The ACAT inhibitor 58035 was provided by Sandoz Pharmaceutical Corp. (East Hanover, NJ, U.S.A.). U18666A was from The Upjohn Co. (Kalamazoo, MI, U.S.A.). FITC-conjugated goat anti-rabbit secondary antibody was obtained from Organon–Teknika (West Chester, PA, U.S.A.). Silicagel 60 TLC plates were from BDH. Lipoprotein-deficient serum (LPDS) was prepared from fetal-calf serum by centrifugation at 150000 ***g*** for 32 h at 10 °C to a density of 1.21 g/ml [28] and dialysed against PBS. All other chemicals were of reagent grade.

Cell culture

CHO-K1 (A.T.C.C. CCL61), CHO 7 and SRD 6 cells (kindly provided by Dr James Metherall, Department of Human Genetics, University of Utah, Salt Lake City, UT, U.S.A.) were grown in monolayers at 37 °C in CO_2/air (1:19) [26,29]. CHO 7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 5 % (v/v) LPDS and 33 μg proline/ml. SRD 6 cells were cultured in DMEM containing 5 % (v/v) LPDS, 1 mM mevalonate, 33 μg proline/ml and 2 μg cholesterol/ml complexed with cyclodextrin. CHO-K1 cells were cultured in DMEM with 5 % (v/v) fetal-calf serum and 33 μg proline/ml. Cells were subcultured in 60-mm or 100-mm dishes in 3 ml and 8 ml of medium respectively. On day 3, cells received fresh medium and experiments were started 18–24 h later (see Figure legends for specific details). 25-Hydroxycholesterol was prepared as a 2.5 mg/ml stock solution in ethanol and added to warm (37 °C) medium to a final concentration of 2.5 μg/ml. 58035 and U18666A were prepared as 1 mg/ml stock solutions in DMSO. CHO 7 and SRD 6 cells that did not receive cyclodextrin–cholesterol complex were incubated with an equivalent amount of cyclodextrin (42 μg/ml or 0.031 mM). This amount of cyclodextrin was insufficient to promote cholesterol efflux [30]. Preliminary experiments indicated that results for control cells receiving no cyclodextrin were indistinguishable from those reported here where cyclodextrin was used in controls.

Analysis of labelled sphingolipids and phospholipids

After labelling with [^{3}H]serine (see Table and Figure legends for specific conditions), cells were rinsed once with ice-cold PBS and scraped into 1 ml of methanol/water (5:4, v/v). Extracts from two dishes were combined and transferred to screwcapped tubes, sonicated with a needle tip at 60 % power setting, on ice, for 30 s and an aliquot was taken for protein determination [31]. Chloroform/methanol (1:1, v/v) and 0.58 % (w/v) NaCl were added to each tube, the tubes were shaken vigorously and the phases were separated by centrifugation at 2000 ***g*** for 5 min at room temperature. The organic phase was extracted twice with 2 ml of methanol/0.58 % (w/v) NaCl/chloroform (45:47:3, by vol.) and dried over anhydrous sodium sulphate. Glycerophospholipids were removed from aliquots of the total lipid extract by base hydrolysis and the remaining radiolabelled SM, glucosylceramide and ceramide were separated by TLC in chloroform/methanol/water (65:25:4, by vol.), identified by fluorography and quantified by liquid scintillation counting [17]. Glycerophospholipids were resolved from an aliquot of the total lipid extract by TLC in chloroform/methanol/acetic acid/water (60:40:4:1, by vol.), identified by exposure to iodine vapour, scraped into vials and the radioactivity was measured by scintillation counting.

Enzyme assays

Serine palmitoyltransferase (SPT) and SM synthase were assayed in total cell membranes prepared in the following manner. Cells were rinsed once, scraped into ice-cold PBS and collected by

centrifugation at 2000 *g* at 4 °C for 5 min. Cells were homogenized in 20 mM Tris/HCl, pH 7.7/10 mM EDTA (buffer A) with 10 strokes in a Dounce homogenizer, centrifuged at 100000 *g* for 1 h and the membrane pellet was resuspended in buffer A to a final concentration of 2–5 mg protein/ml. SM synthase was assayed by a modification [17] of the method of Futerman and Pagano [32]. SPT was assayed as described previously [12].

Immunoblotting and immunoprecipitation of OSBP

Cell monolayers were harvested in ice-cold PBS and collected by centrifugation (2000 *g* at 4 °C for 5 min). Cell pellets were solubilized in 10 mM sodium phosphate, pH 7.4,/150 mM NaCl/2 mM EDTA/2 mM EGTA/10 mM NaF/1 mM sodium pyrophosphate/1 mM β-glycerophosphate/100 μM PMSF containing 2 μg aprotinin/ml, 2.5 μg leupeptin/ml and 0.3 % (w/v) Triton X100 (buffer B) on ice for 15 min followed by centrifugation for 15 min at 10000 *g* in a microcentrifuge. The supernatant, which contained all immunoreactive OSBP, was used for immunoblotting or immunoprecipitation. Protein was determined by the micro-bicinchoninic acid procedure according to the manufacturer's instructions (Pierce).

A glutathione S-transferase–OSBP fusion protein (amino acids 201–309 of rabbit OSBP) was expressed in bacteria, purified and used to immunize rabbits. The antibody (referred to as Ab104) was affinity-purified on a glutathione S-transferase–OSBP fusion protein column. Ab104 reacts with the 97 kDa and 100 kDa OSBP doublet from human, hamster and rabbit sources, and does not appear to cross-react with other OSBP-related proteins (N. D. Ridgway, unpublished work). For immunoblotting, Triton X100 supernatants (10–15 μg) were separated by SDS/6 %-PAGE, transferred to nitrocellulose and incubated for 2 h with affinity-purified Ab104 in 20 mM Tris/HCl, pH 7.4,/150 mM NaCl/0.1 % (v/v) Tween 20/5 % (w/v) skimmed-milk powder [33]. Following incubation with a secondary antibody coupled to horseradish peroxidase, the filter was developed by the enhanced chemiluminescence method according to the manufacturer's instructions (Amersham).

^{32}P-OSBP was immunopreciptated from cell extracts (30–40 μg protein) in 200 μl of buffer B with 1 % (w/v) Triton X-100 and Ab104 for 2 h at 4 °C [33]. Immune complexes were isolated by binding to Protein A-Sepharose, separated by SDS/6 %-PAGE and ^{32}P-OSBP was identified by autoradiography. Immunoprecipitated [^{32}P]OSBP was subjected to tryptic digestion and phosphopeptides were resolved by two-dimensional thin-layer electrophoresis and TLC as described previously [33,34].

Indirect immunofluorescence

Dectection of OSBP by indirect immunofluorescence was performed as described previously [20,21], using affinity-purified Ab104 and FITC-conjugated goat anti-rabbit secondary antibody. Fluorescence microscopy was performed on an Olympus microscope using a 100× oil-immersion objective. Cells were photographed with Kodak TMX400 black-and-white film.

RESULTS

SM synthesis in SRD 6 and lovastatin-treated CHO-K1 cells

SRD 6 cells are a useful model to study the effects of cholesterol deprivation on metabolism of phospholipids and sphingolipids because of reduced *de novo* cholesterol synthesis and LDL uptake, and a requirement for exogenous cholesterol for survival [26]. The effect of cholesterol deprivation on basal and 25-hydroxycholesterol-stimulated SM synthesis (measured by [^{3}H]serine incorporation) in control CHO 7 and SRD 6 cells grown in LPDS with and without exogenous cholesterol was examined (Table 1). It had been reported previously that SRD 6 cells grown in cholesterol-free medium displayed a 40 % reduction in cholesterol compared with CHO 7 cells and cholesterol-supplemented SRD 6 cells [27]. Similarly to wild-type CHO-K1 cells [16], SM synthesis in CHO 7 cells was stimulated by 25-hydroxycholesterol irrespective of the presence of cholesterol in the medium. SRD 6 cells grown in the absence of cholesterol for 24 h showed a 30 % reduction in basal SM synthesis, which was further suppressed by the addition of 25-hydroxycholesterol for 4 h. When SRD 6 cells were supplemented with 2 μg cholesterol/ml, basal SM synthesis remained suppressed relative to controls but activation by 25-hydroxycholesterol (2-fold) was restored. Compared with CHO 7 cells, SRD 6 cells grown in the absence of cholesterol had rates of glucosylceramide synthesis that were increased 2.5-fold, and growth in cholesterol partially corrected this defect (1.7-fold elevated compared with cholesterol-supplemented CHO 7 cells). SRD 6 cells also had lower isotope incorporation into ceramide, but the labelling of phosphatidylserine (PtdSer) and phosphatidylethanolamine (PtdEtn) was similar to controls.

To determine if differences in basal and 25-hydroxycholesterol-stimulated SM synthesis could be related to altered enzyme activity, the activities of SPT and SM synthase, which catalyse the initial and final steps of SM synthesis, respectively, were measured in the particulate (membrane) fraction of CHO 7 and SRD 6 cells grown in cholesterol-free medium for 24 h. The specific activities of SPT and SM synthase in CHO 7 cells were 13.1 ± 5.0 and 28.6 ± 11.1 pmol/min/mg protein respectively (means $\pm$ S.D. for three experiments). This was compared with SPT and SM synthase activities in the particulate fraction of SRD 6 cells of 9.1 ± 3.5 and 17.8 ± 8.1 pmol/min/mg protein, respectively (means $\pm$ S.D. for three experiments). As reported previously for CHO-K1 cells [17], 25-hydroxycholesterol treatment of SRD 6 or CHO 7 cells did not significantly affect the activity of SPT or SM synthase *in vitro*, nor did growth in cholesterol for 24 h (results not shown).

Although it was apparent that cholesterol-starved SRD 6 cells had lost the capacity to increase SM synthesis in response to 25-hydroxycholesterol, the effect on other oxysterol-regulated pathways is not known. To address this question, CHO 7 and SRD 6 cells were cultured with or without cholesterol for 24 h and stimulation of cholesterol esterification by 25-hydroxycholesterol was measured (Table 2). 25-hydroxycholesterol stimulated cholesteryl-ester synthesis 3-fold in untreated CHO 7 cells. Cholesterol supplementation of CHO 7 cells increased basal cholesterol esterification and activation by 25-hydroxycholesterol was still evident. In cholesterol-starved SRD 6 cells, the basal esterification rate was almost undetectable but the presence of 25-hydroxycholesterol caused approx. 4-fold stimulation. In cholesterol-supplemented SRD 6 cells, the basal and 25-hydroxycholesterol-stimulated esterification rates were similar to those in unsupplemented CHO 7 cells.

It was then determined if the phenotype observed in cholesterol-starved SRD 6 cells could be restored in CHO-K1 cells by growth in lipoprotein-free medium with lovastatin, a potent 3-hydroxy-3-methylglutaryl-CoA reductase inhibitor. In these experiments, cells were treated with lovastatin at the concentrations shown in Figure 1 for 12 h, followed by the addition of 25-hydroxycholesterol or a similar volume of ethanol for 4 h, and lipid synthesis was measured by [^{3}H]serine incorporation for 1 h. Lovastatin inhibited [^{14}C]acetate incorporation into cholesterol by > 95 % and caused a 15–20 %

Table 1 Incorporation of label from [^{3}H]serine into sphingolipids and phospholipids in CHO7 and SRD cell lines

Cells were cultured in DMEM with 5% (v/v) LPDS containing no addition (NA, cyclodextrin) or cholesterol (Chol, 2 μg/ml complexed to cyclodextrin) for 24 h. Cells were switched to serine-free DMEM containing 5% (v/v) LDPS with (+) or without (−) 2.5 μg/ml of 25-hydroxycholesterol for 4 h. Cells were pulsed with 7.5 μCi/ml [^{3}H]serine for the final hour of this treatment, harvested and the incorporation of label into lipids was assessed as described in the Materials and methods section. The results (d.p.m./nmol of lipid phosphorus) are the means ± S.D. for three to eight experiments. [a]$P < 0.001$, [b]$P < 0.025$, [c]$P < 0.05$, experimental cells versus cells that were not treated with 25-hydroxycholesterol. [d]$P < 0.01$, [e]$P < 0.05$, [f]$P < 0.025$, [g]$P < 0.001$, experimental cells versus similarily treated CHO 7 cells.

	SM		Glucosylceramide		Ceramide		PtdSer		PtdEtn	
Cell line	−	+	−	+	−	+	−	+	−	+
CHO 7 NA	126 ± 18	190 ± 30[a]	36 ± 6	35 ± 5	142 ± 20	111 ± 18	440 ± 123	419 ± 79	80 ± 18	81 ± 6
CHO 7 + Chol	112 ± 34	203 ± 42[b]	29 ± 6	29 ± 4	112 ± 20	114 ± 9	378 ± 113	443 ± 153	70 ± 19	85 ± 15
SRD 6 NA	88 ± 24[f]	68 ± 27	91 ± 25[g]	59 ± 6	96 ± 17[d]	81 ± 15	347 ± 92	310 ± 137	67 ± 16	74 ± 34
SRD 6 + Chol	64 ± 18	129 ± 29[c]	51 ± 20[e]	51 ± 9	84 ± 20	85 ± 4	351 ± 130	337 ± 144	64 ± 25	63 ± 30

Table 2 Cholesterol esterification in CHO 7 and SRD 6 cells

Cells were cultured with cyclodextrin–cholesterol (+ Chol) or cyclodextin (− Chol) for 24 h as described in the caption of Table 1. Cells were then treated with 25-hydroxycholesterol (25-hydroxycholesterol, 2.5 μg/ml) or solvent (NA, no addition) for 4 h and were pulsed with 100 μM [^{3}H]oleate complexed with BSA for the final hour of treatment, and incorporation of the label into cholesteryl ester was measured [27]. The results are the means ± S.D. of three experiments.

	[^{3}H]Cholesteryloleate formed (pmol/min per mg of protein)	
Cell line	NA	25-Hydroxycholesterol
CHO 7 − Chol	10.0 ± 5.52	34.8 ± 4.6
CHO 7 + Chol	34.4 ± 11.4	50.9 ± 13.8
SRD 6 − Chol	2.7 ± 2.3	10.9 ± 0.8
SRD 6 + Chol	7.4 ± 0.6	25.2 ± 8.4

reduction in cholesterol content at concentrations above 10 μM (results not shown). There was also a pronounced dose-dependent inhibition of both basal and 25-hydroxycholesterol-stimulated SM synthesis. In cells treated with 50 μM lovastatin, basal SM synthesis was reduced by 50% and stimulation by oxysterol was no longer evident. Lovastatin also caused a 50% reduction of [^{3}H]serine incorporation into ceramide compared with a decrease of < 15% for PtdEtn and PtdSer.

Altered OSBP phosphorylation in SRD 6 and lovastatin-treated CHO-K1 cells

We previously reported that 25-hydroxycholesterol stimulated ceramide conversion into SM [17], a reaction that has been localized to the Golgi apparatus [22]. Interestingly, 25-hydroxycholesterol also promoted translocation of OSBP to the Golgi apparatus [20], suggesting that the effects of oxysterol on SM synthesis could be related directly or indirectly to ligand binding and subsequent translocation of OSBP to this organelle. Consequently, we tested whether OSBP expression and localization was affected by cholesterol auxotrophy, and whether aberrant SM synthesis in SRD 6 cells could be correlated with changes in OSBP expression and localization. Figure 2 shows immunoblots (upper panel) and immunoprecipitation of ^{32}P-labelled OSBP (lower panel) from CHO 7 and SRD 6 cells. OSBP migrates as a doublet on SDS/PAGE [18]; the lower mobility of the upper band of the doublet is the result of phosphorylation on at least five serine residues, whereas the lower band is hypophosphorylated and incorporates < 10% of total [^{32}P]P_i [33]. In

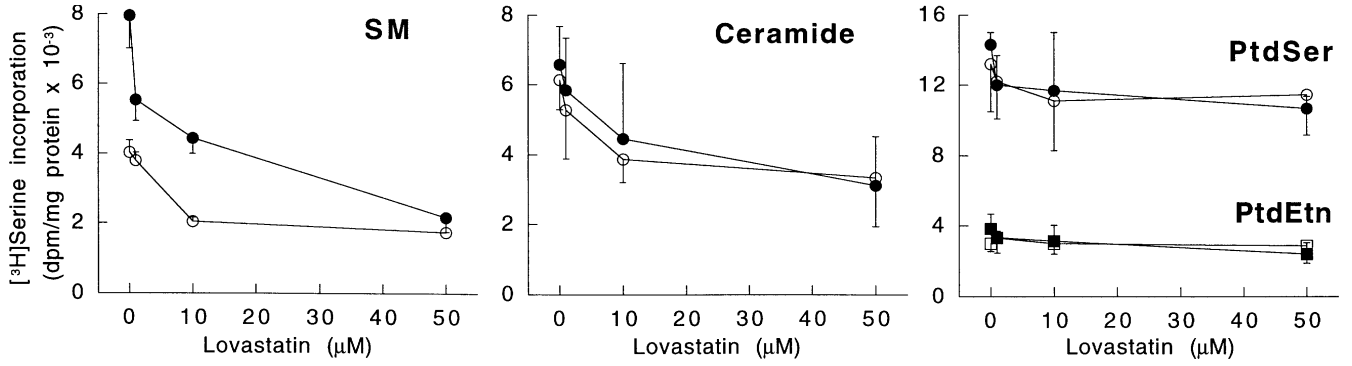

Figure 1 Inhibition of SM synthesis by lovastatin in CHO-K1 cells

CHO-K1 cells were cultured in DMEM with 5% (v/v) LPDS and the indicated concentrations of lovastatin for 12 h. 25-Hydroxycholesterol (2.5 μg/ml, ● or ■) or ethanol (○ or □) was added to the cells for an additional 4 h. During the last hour of oxysterol treatment, the cells were labelled with [^{3}H]serine (7.5 μCi/ml) and incorporation of the label into lipids was assessed as described in the Materials and methods section. The results are the means ± S.D. of three separate experiments.

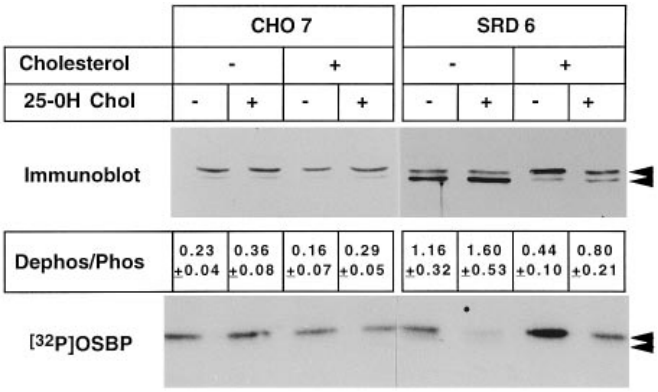

Figure 2 OSBP expression and phosphorylation in CHO 7 and SRD 6 cells

Cells were cultured in DMEM with 5% (v/v) LPDS and either cyclodextrin (no cholesterol) or cyclodextrin–cholesterol (2 μg/ml) for 24 h, followed by 25-hydroxycholesterol (2.5 μg/ml) or ethanol (control) for an additional 4 h. For immunoblot analysis (upper panel), cells were harvested in 0.3% (v/v) Triton X100 buffer and equal amounts of protein (15 μg) were resolved by SDS/6%-PAGE, transferred to nitrocellulose and probed with affinity-purified Ab104. The ratio of dephosphorylated to phosphorylated OSBP (Dephos/Phos) was determined from the amount of the low- and high-molecular-mass isoform, by scanning (means $\pm$ S.D. of four experiments). For [^{32}P]P_i incorporation experiments (lower panel), cells were switched to phosphate-free medium and 100 μCi [^{32}P]P_i/ml was added at the same time as the 25-hydroxycholesterol addition. After 4 h, cells were harvested, radiolabelled OSBP was immunoprecipitated from Triton X-100 extracts (35 μg protein) with Ab104 and resolved on SDS/PAGE as described in the Materials and methods section. The dried gel was exposed to Amersham Hyper film MP for 3 days at −70 °C.

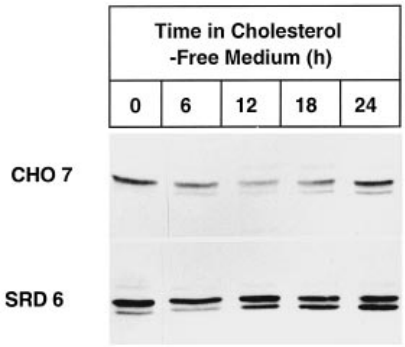

Figure 3 Time course for OSBP dephosphorylation in SRD 6 and CHO 7 cells grown in cholesterol-free medium

CHO 7 and SRD 6 cells were grown in DMEM with 5% LPDS and cholesterol (2 μg/ml complexed to cyclodextrin) for 3 days before the start of experiments. At time 0, cells received cholesterol-free DMEM with 5% (v/v) LPDS for up to 24 h. At the indicated times, cells were harvested and OSBP expression was quantified in Triton X-100 cell extracts by immunoblotting with affinity-purifed Ab104.

CHO 7 cells, the majority (70–80%) of OSBP is in the phosphorylated, high-molecular-mass form. This was not affected by growth in cholesterol or 25-hydroxycholesterol treatment for 4 h. In contrast, the distribution OSBP in SRD 6 cells, grown in the absence of exogenous cholesterol, was shifted to the dephosphorylated, low-molecular-mass form. Growth of SRD 6 cells in cholesterol for 24 h caused a shift to the high-molecular-mass form, so that the distribution resembled CHO 7 cells. Although the proportion of dephosphorylated OSBP in cholesterol-depleted SRD 6 cells was greater, the total amount of phosphorylated protein was similar to CHO 7 because of a 3- to 4-

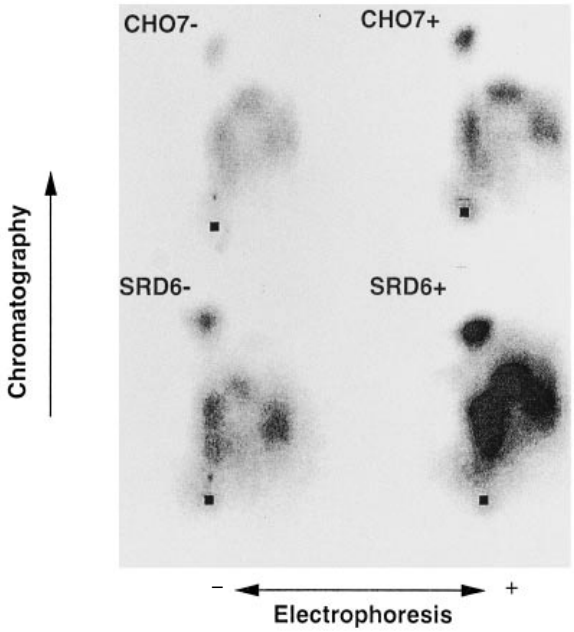

Figure 4 Effect of cholesterol on OSBP phosphopeptide distribution in CHO 7 and SRD 6 cells

Cells were cultured in DMEM with 5% (v/v) LPDS (CHO 7) or medium with 2 μg/ml cholesterol and 1 mM mevalonate (SRD 6) for 3 days before exchanging for DMEM with 5% (v/v) LPDS plus 2 μg cholesterol/ml complexed to cyclodextrin (CHO 7+ and SRD 6+) or cyclodextrin alone (CHO 7− and SRD 6−). After 19 h, cells were switched to phosphate-free medium for 1 h and subsequently pulsed with [^{32}P]P_i (200 μCi/ml) for 4 h. Cells were harvested and ^{32}P-OSBP was immunoprecipitated from equal amounts (200 μg) of the Triton X-100-soluble fraction with Ab104. Immunoprecipitates were subjected to complete tryptic digestion, applied to cellulose thin-layer plates (indicated by the small square) and resolved by two-dimensional thin-layer electrophoresis and chromatography as described in the Materials and methods section. Similar results were observed in two experiments.

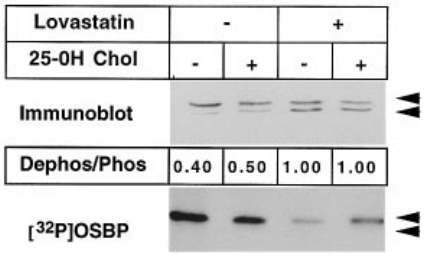

Figure 5 Dephosphorylation of OSBP in lovastatin-treated CHO-K1 cells

CHO-K1 cells were treated essentially as described in the legend to Figure 1. For immunoblot analysis of OSBP (upper panel), 15 μg of a Triton X100 cell extract was resolved on SDS/PAGE, transferred to nitrocellulose and probed with affinity purified Ab104. In immunoprecipitation experiments (lower panel), cells were labelled with [^{32}P]P_i (100 μCi/ml) in phosphate-free DMEM/5% (v/v) LPDS for 1 h before harvesting. Protein (35 μg)from the Triton X-100-soluble fraction of cells was subjected to immunoprecipitation with Ab104, resolved by SDS/PAGE and ^{32}P-OSBP ([^{32}P]OSBP) was visualized by autoradiography after 3 days at −70 °C, using Kodak XAR film.

fold increased expression in SRD 6 cells. OSBP from SRD 6 cells grown with or without cholesterol and treated with 25-hydroxycholesterol displayed a more enhanced shift to the dephosphorylated form compared with CHO 7 cells. Similarly to immunoblotting results, [^{32}P]P_i incorporation into OSBP in CHO

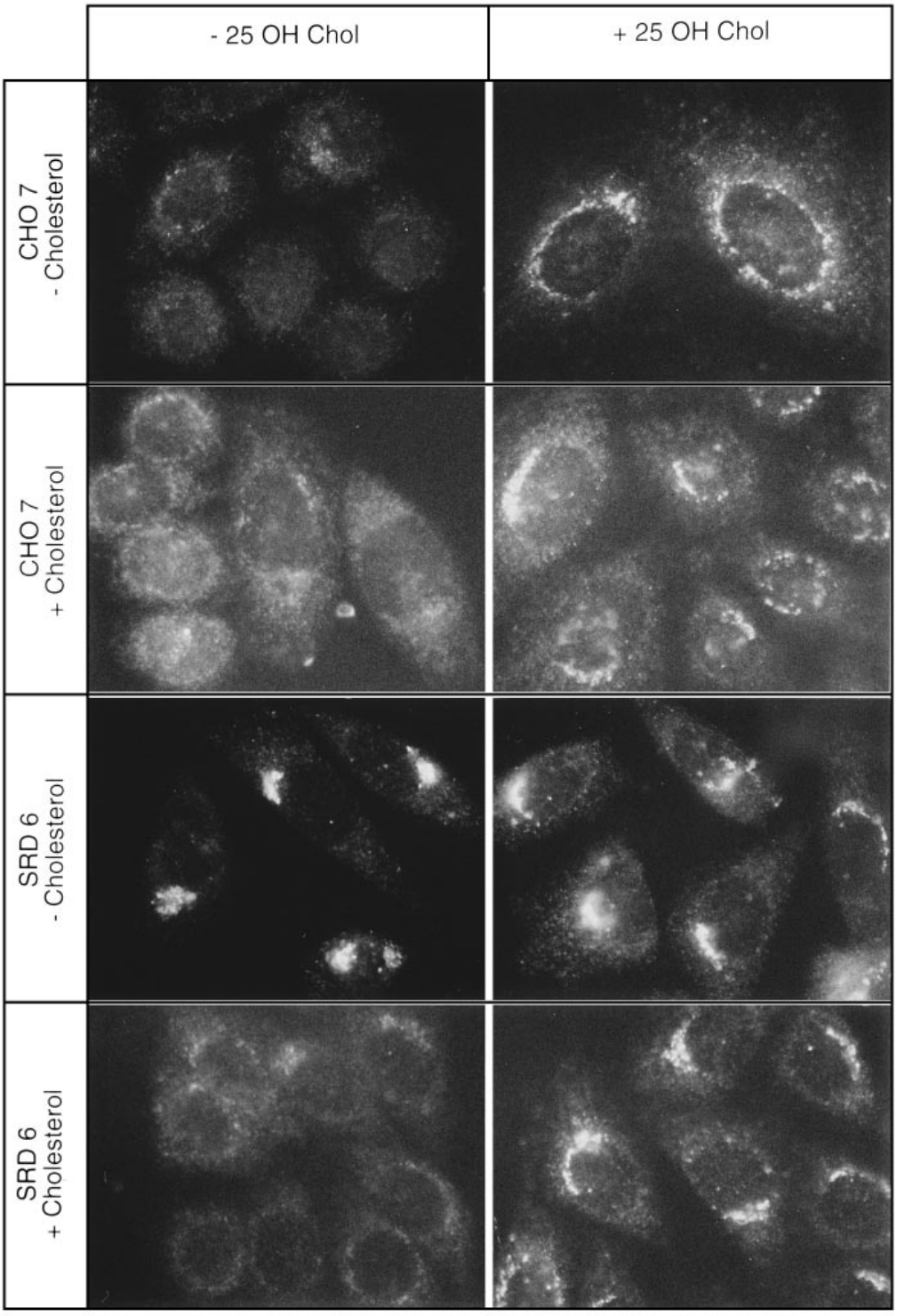

Figure 6 Immunofluorescence localization of OSBP in CHO 7 and SRD 6 cells

Cells were cultured on glass coverslips in DMEM with 5% (v/v) LPDS (CHO 7) or 5% (v/v) LPDS with 1 mM mevalonate and cholesterol (2 μg/ml) complexed to cyclodextrin (SRD 6) for 3 days before the start of experiments. Cells then received fresh DMEM with 5% (v/v) LPDS with cholesterol (2 μg/ml) complexed to cyclodextrin or cyclodextrin alone for 24 h. During the last 4 h, cells were treated with 25-hydroxycholesterol (2.5 μg/ml) or solvent (ethanol). Cells were processed for immunofluorescence using affinity-purified Ab104 as described in the Materials and methods section.

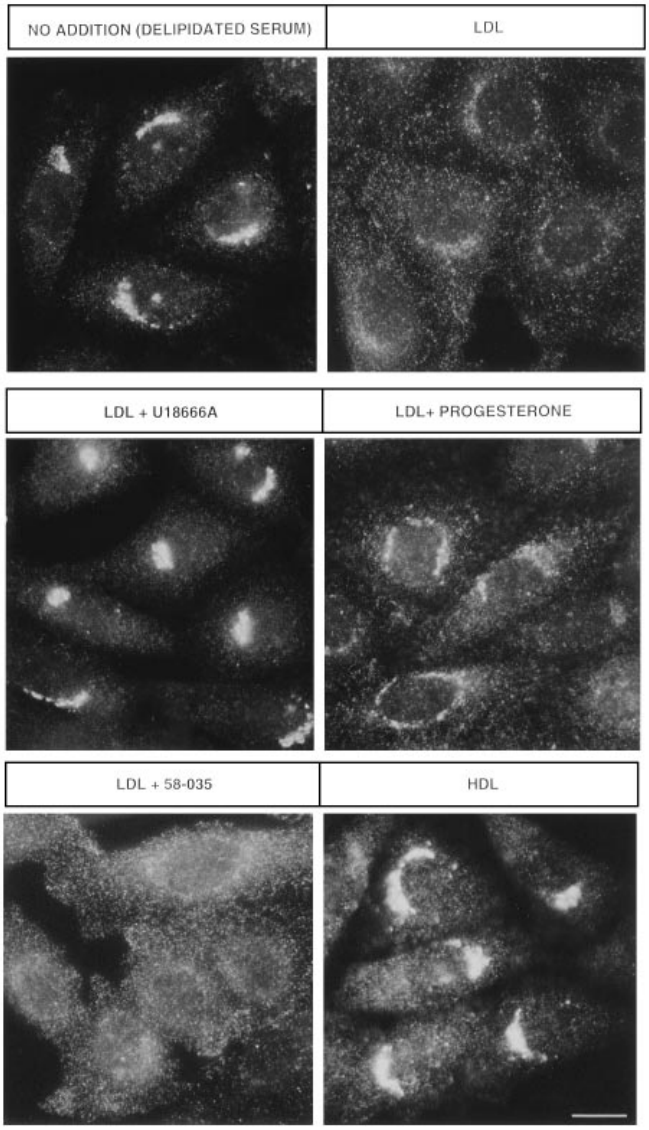

Figure 7 Effect of LDL cholesterol on the distribution of OSBP in CHO-K1 cells

CHO-K1 cells were cultured on glass coverslips in DMEM plus 5% (v/v) LPDS with either no addition, LDL (50 μg/ml), LDL (50 μg/ml) with U18666A (1 μg/ml), LDL (50 μg/ml) with progesterone (1 μg/ml), LDL (1 μg/ml) with 58035 (2 μg/ml) or high-density lipoprotein (HDL) (50 μg/ml) for 24 h. The cells were processed for indirect immunofluorescence using affinity-purified Ab104 followed by a FITC-goat anti-rabbit secondary antibody.

7 cells was similar under all conditions (Figure 2, lower panel). OSBP from SRD 6 cells grown without cholesterol had a similar phosphorylation level to CHO 7 (reflecting the high proportion in the dephosphorylated state), and growth of SRD 6 cells in cholesterol increased [^{32}P]P_i incorporation by 60–70%. Consistently with immunoblot analysis, 25-hydroxycholesterol treatment of SRD 6 cells reduced [^{32}P]P_i incorporation into OSBP in both cholesterol-depleted and -supplemented SRD 6 cells. The results shown in Figure 2 are not the result of a decrease in total protein phosphorylation due to cholesterol depletion, since [^{32}P]P_i incorporation into total protein was actually 31% greater in SRD 6 cells grown in the absence of exogenous cholesterol (average of three experiments).

A time course for changes in SRD 6 phosphorylation status, measured by distribution of the two isoforms following growth in lipoprotein-free medium without exogenous cholesterol, is shown in Figure 3. Growth in cholesterol-free medium had little effect on the isoform distribution of OSBP in CHO 7 cells, except for a slight shift to the low-molecular-mass form at 24 h. Initially, OSBP from SRD 6 cells had a similar isoform distribution as CHO 7 cells, but after 12 h in cholesterol-free medium there was a pronounced shift to the dephosphorylated, lower-molecular-mass isoform that was maintained until 24 h.

OSBP can be resolved into five major phosphopeptides by tryptic digestion [33]. To determine whether phosphorylation of individual sites in OSBP is cholesterol-sensitive, CHO 7 and SRD 6 cells were grown in the presence or absence of cholesterol for 24 h, labelled with [^{32}P]P_i, digested with trypsin and phosphopeptides were resolved in two dimensions (Figure 4). Phosphopeptide maps for OSBP immunoprecipitated from CHO 7 cells showed the presence of five major phosphopeptides, the proportions of which were not affected by cholesterol in the medium. In contrast, OSBP from SRD 6 cells grown in cholesterol for 24 h had increased labelling of all phosphopeptides compared with cholesterol-depleted cells. The phosphopepetide map for OSBP from SRD 6 cells grown in cholesterol was similar to that for CHO 7 cells but the incorporation of [^{32}P]P_i into all peptides was increased.

We examined whether inhibition of basal and 25-hydroxycholesterol-stimulated SM synthesis in lovastatin-treated CHO-K1 cells was also accompanied by OSBP dephosphorylation (Figure 5). Lovastatin (50 μM) treatment for 12 h resulted in a shift in distribution of OSBP to the lower-molecular-mass, dephosphorylated form (immunoblot, upper panel). OSBP dephosphorylation in lovastatin-treated cells was also demonstrated by [^{32}P]P_i incorporation and immunoprecipitation (lower panel). 25-hydroxycholesterol treatment for 4 h in the presence or absence of lovastatin did not affect OSBP.

Influence of cholesterol on intracellular localization of OSBP

OSBP overexpressed in CHO-K1 cells undergoes translocation from a cytoplasmic/vesicular compartment to the Golgi complex in the presence of ligand [20,21], an event that is dependent on the pleckstrin-homology domain [21]. The effect of cholesterol depletion on translocation and localization of endogenous OSBP to these compartments was monitored in SRD 6 and CHO 7 cells by indirect immunofluorescence. Untreated CHO 7 cells grown in the presence or absence of cholesterol for 24 h displayed cytoplasmic OSBP, as well as diffuse staining of small vesicles around the nucleus (Figure 6). Addition of 25-hydroxycholesterol for 4 h caused OSBP translocation to structures around, or localized to, one pole of the nucleus. This was similar to results with the overexpressed protein and was shown to correspond to the Golgi apparatus [20]. In contrast to CHO 7 cells, OSBP in SRD 6 cells grown in the absence of cholesterol displayed constitutive Golgi localization that was not affected by 25-hydroxycholesterol. Growth of SRD 6 cells in cholesterol for 24 h caused OSBP to return to the cytoplasmic/vesicular compartment, where it translocated to the Golgi apparatus in response to 25-hydroxycholesterol. The localization of the Golgi-specific marker FITC-lentil lectin [20] was not affected by cholesterol or 25-hydroxycholesterol supplementation or the SRD 6 mutation (results not shown).

SRD 6 cells are an extreme model of cholesterol depletion, since both *de novo* synthesis and lipoprotein uptake are impaired [26], and the cells rely on non-physiological uptake of cholesterol complexed to cyclodextrins. Also, OSBP distribution in CHO 7

cells may not be representative of the normal situation since these cells are adapted for growth in lipoprotein-free conditions [29]. Accordingly, the sensitivity of OSBP localization to alterations in cholesterol uptake and trafficking was tested in wild-type CHO-K1 cells (Figure 7). Similarly to results with cholesterol-depleted SRD6 cells, OSBP in CHO-K1 cells cultured in LPDS for 24 h was predominantly in the Golgi apparatus. OSBP in cells cultured in LPDS plus human LDL (50 μg/ml), but not high-density lipoprotein (50 μg/ml), was localized to diffusely scattered small vesicles, but also some faint Golgi staining was evident. The LDL-mediated release of OSBP from the Golgi complex was prevented by simultaneous addition of two compounds that inhibit release of cholesterol from lysosomes, U18666A (1 μg/ml) or progesterone (1 μg/ml) [35]. Progesterone appeared to be slightly less effective in preserving Golgi staining of OSBP. Inhibition of LDL cholesterol esterification with the ACAT inhibitor 58035 did not affect OSBP localization in cells cultured in LDL. The phosphorylation status of OSBP in CHO-K1 cells (measured by immunoblotting) was not affected by growth in LDL or LPDS (results not shown).

DISCUSSION

A somatic cell mutant in cholesterol synthesis was used to explore the regulation of cholesterol and SM synthesis by 25-hydroxycholesterol and OSBP. We demonstrated that activation of SM synthesis by 25-hydroxycholesterol was repressed in cholesterol-depleted cells. This was accompanied by dephosphorylation and constitutive localization of OSBP to the Golgi apparatus. In the case of SRD 6 cells, this was reversed if non-lipoprotein cholesterol was included in the culture medium. A similar observation was made in CHO-K1 cells, where egress of LDL cholesterol from the lysosome was required to mediate OSBP release from the Golgi complex. Collectively, these results show that OSBP phosphorylation and intracellular movement are regulated by cholesterol content and trafficking, and implicate OSBP as a regulator of sterol and SM synthesis at the Golgi complex.

SRD 6 cells have reduced or absent mRNA and activity for multiple enzymes in the sterol synthetic pathway [26] and virtually no *de novo* synthesis of cholesterol due to incomplete processing of SREBP to its mature, nuclear localized form [25]. In addition to defects in cholesterol synthesis, basal SM synthesis, but not SM mass [27], was reduced by 30–50 % in SRD 6 cells grown in either the presence or absence of exogenous cholesterol. This could be attributed to a similar reduction in the *in vitro* activity of two key enzymes in the pathway, but whether SREBP is directly involved in the expression of these two enzymes is uncertain. The mRNA for an SPT isoform was not regulated in parallel with cholesterol and fatty acid biosynthetic enzymes in keratinocytes, indicating that SREBP was not involved under conditions that acutely affect cholesterol synthesis [36]. Because basal SM synthesis could not be stimulated by exogenous cholesterol in SRD 6 cells, it would appear that an intact *de novo* pathway for cholesterol synthesis and regulation, or a non-sterol intermediate, is required for full activity of the SM biosynthetic pathway. This was confirmed in CHO-K1 cells by lovastatin-mediated inhibition of cholesterol synthesis, which resulted in a marked inhibition of SM synthesis but not synthesis of PtdSer or PtdEtn. These effects of lovastatin were not observed in CaCo-2 cells [14], which could be attributed to incomplete inhibition of cholesterol synthesis or a cell-specific effect.

Although a fully active pathway for cholesterol synthesis and regulation appeared to be necessary for SM synthesis in CHO cells, the SM synthesis defect in SRD 6 cells could also result from aberrant phosphatidylcholine (PtdCho) and fatty acid biosynthesis metabolism. It has been reported previously that SRD 6 cells had lower incorporation of [^{3}H]choline into PtdCho, in addition to SM, and reduced PtdCho mass [27]. Thus the lower incorporation of [^{3}H]serine into SM demonstrated in the present study may simply reflect restricted availability of PtdCho for donation of the phosphocholine headgroup to ceramide. In addition to effects on cholesterol synthesis, the SREBP-processing defect in SRD 6 cells also resulted in a 75 % reduction in acetate incorporation into fatty acids (M. K. Storey and N. D. Ridgway, unpublished work), which was consistent with the role of SREBPs in transcription of fatty acid synthase and acetyl-CoA carboxylase [37,38]. In SRD 6 cells, exogenous oleate was found to stimulate the activity of CTP : phosphocholine cytidylyltransferase [16], the rate-limiting enzyme for PtdCho synthesis, and to restore PtdCho synthesis to control values. This suggests that lower basal SM synthesis in SRD 6 can be traced back to defective PtdCho synthesis due to fatty acid auxotrophy. Thus the pivotal role of SREBP in sterol and fatty acid homoeostasis also extends to the regulation of PtdCho and SM.

Distinct from the effects of the SRD 6 mutation on basal SM synthesis was the lack of response to 25-hydroxycholesterol upon cholesterol depletion and its restoration by exogenous cholesterol. Labelling studies in CHO-K1 cells suggested that the primary site of action of 25-hydroxycholesterol was conversion of ceramide to SM. However, the activity of SM synthase *in vitro* was unaffected by 25-hydroxycholesterol or growth in cholesterol. SM synthase activity is unlikely to be directly affected by cholesterol, since reconstitution in PtdCho vesicles containing 15 mol % of cholesterol did not alter activity [39]. The amount of ceramide available to this enzyme may be affected by cholesterol or 25-hydroxycholesterol. Cholesterol-depleted fibroblasts displayed enhanced fluorescent bleaching of *N*-[7-(4-nitrobenzo-2-oxa-1,3-diazol)]-6-aminohexanoylsphingosine ('C_6-NBD-ceramide') in the Golgi apparatus [40]. Whereas this result does not indicate that less ceramide is present in the cholesterol-depleted Golgi, it does suggest that cholesterol alters the lipid environment and perhaps the deposition of ceramide in the Golgi complex. In the present study, SM synthase was assayed with labelled C_6-ceramide and thus changes in activity due to endogenous substrate availability were not detected.

CHO-K1 and SRD 6 cells grown in delipidated or cholesterol-free medium resulted in OSBP localization to the Golgi apparatus, an event that was reversed with LDL or non-lipoprotein cholesterol respectively. These results are consistent with a model where OSBP cycles between these compartments and cholesterol depletion acts to effectively 'lock' OSBP into the Golgi apparatus. CHO 7 cells did not display significant Golgi localization of OSBP when grown in delipidated medium (Figure 6) probably because of long-term growth adaptation to lipoprotein-free conditions [29]. The Golgi apparatus is known to contain significant amounts of cholesterol [2,24,41]; the cholesterol content of the Golgi elements is affected by uptake and catabolism of LDL [24,40], and cholesterol appears to transit via the Golgi complex [23]. It is possible that OSBP translocation is regulated by Golgi cholesterol content and it serves as a signal or sensor at this organelle. The previous finding that OSBP was translocated to the Golgi complex in response to exogenous 25-hydroxycholesterol would seem at odds with Golgi localization under conditions of cholesterol depletion. However, 25-hydroxycholesterol causes cellular cholesterol depletion by inhibition of synthesis and activation of cholesterol esterification [18], presumably by enhanced cholesterol transport from the plasma membrane to the endoplasmic reticulum [42]. The net effect would be similar to that seen in cholesterol-depleted SRD 6 or

CHO-K1 cells. This raises the possibility that binding of an exogenous oxysterol ligand by OSBP (or a related homologue) promotes rapid movement of cholesterol to regulatory sites in the endoplasmic reticulum. Our finding that OSBP is primarily in the cytoplasmic/vesicular compartment of cells grown in cholesterol-replete conditions (i.e. LDL or cholesterol–cyclodextrin) also implies that regulatory oxysterols are not produced intracellularly in insignificant amounts to stimulate OSBP. It would appear that OSBP localization to the Golgi apparatus is regulated by exogenous oxysterols and changes in cellular cholesterol content and localization.

In SRD 6 cells, localization of OSBP to the Golgi apparatus also coincided with loss of stimulation of SM synthesis by 25-hydroxycholesterol. CHO-K1 cells grown in delipidated medium, a condition where OSBP is localized to the Golgi (Figure 7), also had a diminished response to 25-hydroxycholesterol [17]. We can assume that OSBP localized to the Golgi apparatus is either unable to bind 25-hydroxycholesterol or is competent for binding but unable to transduce the signal to downstream effectors. Although this is the case for stimulation of SM synthesis, it does not appear to hold true for activation of ACAT, since cholesterol esterification in SRD 6 cells was stimulated by 25-hydroxycholesterol to the same degree as control cells (3-fold, see Table 2). It is important to note that esterification in SRD 6 cells was also 2 to 4-fold lower when compared with similarly treated CHO 7 cells. Since it has been shown that 25-hydroxycholesterol acts as a direct activator of ACAT in a cell-free system [43], it is possible that stimulation by 25-hydroxycholesterol occurs in the endoplasmic reticulum and does not involve OSBP or events at the Golgi apparatus.

In SRD 6 and lovastatin-treated CHO-K1 cells, cholesterol depletion was accompanied also by dephosphorylation of OSBP. 25-Hydroxycholesterol promoted more extensive dephosphorylation of OSBP, which could have resulted from further diminution of cholesterol levels due to activation of cholesterol esterification. It is likely that abnormal OSBP phosphorylation in cholesterol-depleted cells is a secondary consequence of cholesterol depletion and Golgi localization. The absolute level of phosphorylated OSBP was similar in CHO 7 and cholesterol-depleted SRD 6 cells (see Figure 4), as were the phosphopepetide maps. If phosphorylated OSBP was required for a specific function, sufficient amounts should have been available. A possible explanation is that an OSBP kinase or phosphatase is sequestered in the cellular compartments which OSBP cycles between. When this cycle is interrupted, OSBP becomes constitutively localized to one compartment and its phosphorylation is altered. The Golgi apparatus is the likely site for phosphorylation of OSBP as indicated by sensitivity to brefeldin A and the presence of a kinase in Golgi-enriched membranes that phosphorylated OSBP *in vitro* [32]. In cholesterol-depleted SRD cells, OSBP appears to enter the Golgi apparatus but its subsequent phosphorylation is partially blocked and it is unable to relocalize to the vesicular/cytoplasmic compartment. Interestingly, treatment of cells with 25-hydroxycholesterol ([33]; Figures 2 and 5), LDL and delipidated medium did not affect the phosphorylation of OSBP in CHO-K1 cells. This implies that only chronic cholesterol starvation is sufficient to promote dephosphorylation of OSBP.

The finding that OSBP and oxysterol are involved in SM and cholesterol metabolism at the Golgi apparatus has some interesting parallels in studies of the *Saccharomyces cerevisiae* OSBP homologue, kes1p. Deletion of *KES 1* by-passed a lethal sec14^{ts} mutation, which results in the accumulation of PtdCho in the Golgi apparatus and cessation of secretion. It is thought that kes1p, which encompasses only the ligand-binding domain of OSBP [44], is a negative regulator of sec14p and is activated when Golgi diglyceride levels decrease [45,46]. OSBP could have a related function by directly or indirectly regulating formation and consumption of diglyceride and PtdCho respectively through changes in SM synthesis. The consequences of this could be altered vesicle budding at the Golgi apparatus induced by the non-bilayer properties of diglyceride [47], and/or activation of protein kinase C [48]. In addition to changing diglyceride levels, activation of SM synthesis would also consume ceramide, which has been demonstrated in 25-hydroxycholesterol-treated CHO-K1 cells [17]. Ceramide is reported to have myriad effects on cell signalling pathways [49], and short-chain analogues of ceramide have been shown to inhibit viral glycoprotein transport through the Golgi [50] and to modify the sensitivity of cells to brefeldin A [51]. As suggested previously, changing SM synthesis could have profound effects on Golgi function by altering levels of these bioactive lipids [52]. Although the precise function of OSBP has yet to be elucidated, regulation of its localization and phosphorylation by cholesterol, and subsequent effects on SM synthesis, indicate an important role in metabolism or trafficking of cholesterol and SM.

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