Altered regulation of cholesterol and cholesteryl ester synthesis in Chinese-hamster ovary cells overexpressing the oxysterol-binding protein is dependent on the pleckstrin homology domain

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Oxysterol-binding protein (OSBP) is a high-affinity receptor for a variety of oxysterols, such as 25-hydroxycholesterol, that down-regulate cholesterol synthesis and stimulate cholesterol esterification. To examine a potential role for OSBP in regulating cholesterol metabolism, we stably overexpressed this protein in Chinese-hamster ovary (CHO)-K1 cells. Compared with mock-transfected controls, several cell lines overexpressing wild-type OSBP (CHO-OSBP) displayed a 50 % decrease in cholesteryl ester synthesis when cultured in medium with delipidated serum, 25-hydroxycholesterol or low-density lipoprotein (LDL). CHO-OSBP cells showed a 40–60 % decrease in acyl-CoA:cholesterol acyltransferase activity and mRNA, a 50 % elevation in mRNA for three sterol-regulated genes [LDL receptor, 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase and HMG-CoA synthase], and an 80 % increase in [¹⁴C]acetate incorporation into

cholesterol. CHO-K1 cells overexpressing two OSBP mutants with a complete or N-terminal deletion of the pleckstrin homology (PH) domain had cholesterol esterification and synthesis rates that were similar to those shown by mock-transfected controls. Unlike wild-type OSBP, both PH domain mutants displayed diffuse cytoplasmic immunofluorescence staining and did not translocate to the Golgi apparatus in the presence of 25-hydroxycholesterol. CHO-K1 cells overexpressing OSBP have pronounced alterations in cholesterol esterification and synthesis, indicating a potential role for this receptor in cholesterol homeostasis. The phenotype observed in cells overexpressing OSBP is dependent on the PH domain, which appears to be necessary for ligand-dependent localization of OSBP to the Golgi apparatus.

INTRODUCTION

Oxysterols have a wide range of effects on cellular physiology that are poorly defined in terms of precise intracellular targets and primary versus non-specific responses (reviewed in [1,2]). An extensively studied aspect of oxysterol action is the putative role of these molecules in regulating cholesterol metabolism [3]. Oxysterol treatment of cultured cells reproduces many of the regulatory responses of low-density lipoprotein (LDL)-derived cholesterol such as transcriptional suppression of sterol-regulated genes, stimulation of acyl-CoA:cholesterol acyltransferase (ACAT) and enhanced degradation of 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase [4]. One hypothesis for the action of oxysterols is that they are generated in cells when the cholesterol content is elevated and suppress sterol synthesis by interaction with a regulatory protein(s). Kandutsch and co-workers [5,6] identified a high-affinity (K_D 10 nM) oxysterol-binding protein (OSBP) in the cytosol of cultured cells and various tissues. It was postulated that OSBP mediated some regulatory effects of oxysterols on cholesterol metabolism. This conclusion was based primarily on evidence of a positive correlation between oxysterol suppression of cholesterol synthesis and HMG-CoA reductase activity in cultured cells, and affinity for OSBP [5,7]. cDNA cloning of human [8] and rabbit [9] OSBPs revealed highly conserved proteins that migrated on SDS/PAGE as a doublet of 97 and 101 kDa [9,10]. Purified [10] or overexpressed OSBP from COS cells [11] had a native molecular mass consistent with a homodimer. Immunofluo-

rescence localization in overexpressing Chinese-hamster ovary (CHO)-K1 cells indicated that OSBP was predominately cytoplasmic or vesicle-associated in the absence of oxysterol, but underwent rapid localization to the Golgi apparatus when treated with 25-hydroxycholesterol [11]. The N-terminal region of OSBP was shown to be important for localization to the Golgi apparatus [11], and contains a pleckstrin homology (PH) domain, a motif with a putative role in intracellular signalling [12,13].

A family of OSBP homologues has been identified in *Saccharomyces cerevisiae* [14]. Yeast harbouring double or triple deletions of members of this gene family displayed cold-sensitive growth and nystatin resistance, as well as small cumulative reductions in ergosterol synthesis [14]. Interestingly, deletion of one member of this OSBP-related family (KES 1) was found to bypass a SEC 14 temperature-sensitive mutation, suggesting that the kes 1 protein may be involved in Golgi function and vesicle trafficking [15]. Because of its apparent association with the Golgi/vesicular pathway, OSBP and related proteins could be involved in aspects of cholesterol or oxysterol trafficking and thereby modify downstream regulatory events by affecting the sterol content at regulatory sites in the endoplasmic reticulum.

To assess the role of OSBP in regulation of cellular cholesterol synthesis and esterification, we studied cholesterol homeostasis in CHO-K1 cells overexpressing wild-type rabbit OSBP. Overexpression of OSBP resulted in a decrease in ACAT activity and mRNA and elevated mRNA for sterol-regulated genes and cholesterol synthesis in cells grown in lipoprotein-free medium. Additional evidence is presented demonstrating that this altered

Abbreviations used: ACAT, acyl-CoA:cholesterol acyltransferase; CHO, Chinese-hamster ovary; DMEM, Dulbecco's modified Eagle's medium; LDL, low-density lipoprotein; FCS, fetal-calf serum; HMG, 3-hydroxy-3-methylglutaryl; OSBP, oxysterol-binding protein; PH, pleckstrin homology.

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regulatory phenotype observed in cells overexpressing wild-type OSBP requires the PH domain.

MATERIALS AND METHODS

Materials

25-Hydroxycholesterol and cholesterol were purchased from Steraloids (Wilton, NH, U.S.A.). Other oxysterols, fatty acid-free BSA and oleate were from Sigma Chemical Co. [α - 32 P]dATP, [9,10- 3 H]oleate, [3 H]25-hydroxycholesterol, [1- 14 C]acetate and [1- 14 C]oleoyl-CoA were from Dupont-NEN. Silica-gel G TLC plates were from BDH. Tissue-culture reagents were from Gibco-BRL. FITC-labelled rabbit anti-mouse IgG was from Organon Teknika (Westchester, PA, U.S.A.). Goat anti-mouse IgG-horseradish peroxidase conjugate was purchased from Bio-Rad.

Cell culture and transfections

CHO-K1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with 5% fetal-calf serum (FCS) (medium A). CHO-K1 cells (100 mm diam. dishes) were transfected with 10 μ g of wild-type or mutant OSBP cDNA in pCMV and 1 μ g of pSV3Neo by the calcium phosphate precipitation method [11]. Mock (control)-transfected cells received equivalent amounts of pCMV and pSV3Neo. Clones resistant to 600 μ g of G418 (Geneticin)/ml were selected and expression of OSBP was determined by immunoblotting (see below). Stock cultures of OSBP-expressing and mock transfected cells were maintained in medium A containing 350 μ g G418 (Geneticin)/ml, but were subcultured for experiments in medium A without G418. Cells were cultured in DMEM with 5% lipoprotein-free FCS 18 h before the start of experiments. OSBP expression was routinely monitored by immunoblotting and immunofluorescence to ensure that 90% of the cells were stably overexpressing.

COS 7 cells were cultured in DMEM containing 10% FCS. COS 7 cells were transiently transfected with wild-type and mutant OSBP cDNAs by the DEAE-dextran method [16] and harvested 48 h later.

Site-directed mutagenesis

The 1230 bp *Sma*I-*Xba*I fragment of the rabbit OSBP cDNA [9] was subcloned into pAlter-1 and mutagenized according to the manufacturer's instructions (Altered Sites II system; Promega). Two deletion mutants of OSBP were generated; the entire PH domain was deleted (OSBP Δ PH, amino acids 92–182) and the N-terminal region of this domain was removed (OSBP Δ N-PH, amino acids 92–125). Both mutations were confirmed by sequencing.

Immunoblotting and immunofluorescence

Stably overexpressing CHO-K1 cells were harvested in ice-cold PBS and collected by centrifugation (2000 *g* for 5 min at 4 °C). 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 μ M PMSF/aprotinin (2 μ g/ml)/leupeptin (2.5 μ g/ml)/0.3% (w/v) Triton X-100 (buffer A) on ice for 20 min, followed by centrifugation for 15 min at 4 °C and 10000 *g* (r_{av} 6 cm). The supernatant fraction, which contained all immunoreactive OSBP, was collected and proteins separated on SDS/6%-PAGE and transferred to nitrocellulose filters. Filters were probed with OSBP monoclonal antibody 11H9 [11] (kindly provided by Dr.

Joseph Goldstein, University of Texas Southwestern Medical Center, Dallas, TX, U.S.A.) followed by goat anti-mouse IgG coupled to horseradish peroxidase. The filter was developed by the enhanced chemiluminescence technique according to the manufacturer's instructions (ECL; Amersham). Indirect immunofluorescence localization of OSBP was as previously described [11] using an Olympus microscope and 100 $\times$ -magnification oil-immersion objective.

[3 H]25-Hydroxycholesterol binding assays

Monolayers of COS 7 cells overexpressing wild-type and mutant OSBP were washed once with cold PBS, scraped into PBS and collected by centrifugation (2000 *g* for 5 min). Cells were homogenized in 10 mM Hepes (pH 7.4)/50 mM KCl/5 mM dithiothreitol/1 mM EDTA/pepstatin A (0.5 μ g/ml)/aprotinin (0.5 μ g/ml)/50 μ M leupeptin/0.6 mM PMSF/10 mM NaF/1 mM β -glycerophosphate/1 mM sodium pyrophosphate by 25 passages through a 23-gauge needle and sedimented at 100000 *g* (r_{av} 6 cm) for 45 min at 4 °C. The cytosol fraction was collected and equivalent amounts of protein (0.3 mg/ml) were assayed for specific binding of [3 H]25-hydroxycholesterol (199 d.p.m./fmol) by the charcoal/dextran method [5].

Cholesterol esterification and ACAT assays

Measurement of cholesterol esterification in monolayers of CHO-K1 cells was performed using 100 μ M [3 H]oleate/BSA in the culture medium [17]. [3 H]Oleate-labelled cholesteryl ester, triacylglycerol and phospholipid were separated by TLC and quantified by liquid-scintillation counting.

For *in vitro* ACAT assays, cells were harvested in cold PBS and homogenized in 20 mM Tris/HCl (pH 7.7)/1 mM EDTA (Tris/EDTA buffer) by 20 passages through a 23-gauge needle [18]. Homogenates were subjected to centrifugation at 4 °C and 100000 *g* (r_{av} 6 cm) for 1 h, and the membrane pellet was suspended in Tris/EDTA buffer. Assays contained 25–50 μ g of protein and 2 mg of fatty-acid-free BSA/ml in 90 μ l of Tris/EDTA buffer. Samples were preincubated for 2 min at 37 °C and the reaction was initiated by the addition of 10 μ l of 0.5 mM [1- 14 C]oleoyl-CoA (100–120 d.p.m./pmol). The reaction was terminated after 10 min by the addition of 2 ml of chloroform/methanol (1:1, v/v). Lipids were extracted, separated by TLC in hexane/diethyl ether/acetic acid (90:30:1, by vol.) and the plates were briefly exposed to iodine vapour. Cholesteryl esters were scraped into vials and radioactivity was quantified by liquid-scintillation counting.

Other methods

mRNA for HMG-CoA reductase, HMG-CoA synthase, ACAT and the LDL receptor was quantified by S1 nuclease protection assays [19]. The ACAT S1 probe corresponding to nucleotides 25–339 of the hamster sequence [20] was obtained by PCR CHO-K1 cDNA. Cholesterol synthesis in mock- and OSBP-transfected CHO-K1 cells was measured by [1- 14 C]acetate (7.5 μ Ci/ml, 55 mCi/mmol) incorporation for 2 h. Cells and medium were pooled and saponified in 50% ethanol/0.7 M KOH [21]. Cholesterol and lanosterol were resolved by TLC in light petroleum ether (b.p. 38.1–52.5 °C)/diethyl ether/acetic acid (60:40:1, by vol.) and identified by fluorography and co-migration with authentic standards.

RESULTS

Cholesterol esterification in cells overexpressing OSBP

25-Hydroxycholesterol is a potent activator of cholesterol esterification [22]. We assessed whether stably overexpressing OSBP in CHO-K1 cells affected basal and 25-hydroxycholesterol-stimulated cholesterol esterification compared with mock-transfected cell lines. OSBP was expressed at low levels in three mock-transfected CHO-K1 cells (M1, M2 and M3) and is evident as a faint band only after prolonged overexposure of immunoblots (not seen in Figure 1A). On the basis of results with the monoclonal antibody and two polyclonal OSBP antibodies, it was estimated that overexpression was approx. 25-fold in OSBP 7 and 16 cells and 15-fold in OSBP 18 cells. 25-Hydroxycholesterol treatment caused a time-dependent stimulation of [3 H]oleate incorporation into cholesteryl ester in all six cell lines that reached a maximum by 2 h (Figure 1B). However, cholesterol esterification in the three OSBP-transfected cells was approximately one-half that observed in mock-transfected control cells at all time points. [3 H]Oleate incorporation into triacylglycerol was inhibited by 25-hydroxycholesterol treatment in all six cell lines (Figure 1C). Basal triacylglycerol synthesis in untreated cells, and incorporation at 2 and 4 h, was reduced relative to the control in two of the OSBP-expressing lines.

The effect of different oxysterols on cholesterol esterification, triacylglycerol synthesis and phospholipid synthesis was examined more closely in one of the CHO-K1 cell lines overexpressing OSBP (clone 16, hereafter referred to as 'CHO-OSBP cells') and compared with mock-transfected control cells (clone M3). As seen in Table 1, CHO-OSBP cells grown in lipoprotein-free serum (NA, no addition) had significantly decreased cholesterol esterification compared with mock-transfected cells. Treatment with a variety of oxysterols for 4 h increased cholesterol esterification to various extents, with the greatest stimulation (4-fold) afforded by 25-hydroxycholesterol. In all cases, CHO-OSBP cells had esterification rates that were 40–50% of control values. Triacylglycerol synthesis in CHO-OSBP and control cells was inhibited to various extents by oxysterol treatment, with the largest suppression by 25- and 20-hydroxycholesterol. Triacylglycerol labelling in CHO-OSBP was consistently decreased compared with control cells, but only in the case of 20- and 22(S)-hydroxycholesterol-treated cells was this significant. [3 H]Oleate incorporation into total phospholipids was similar for both cell lines and was not affected by oxysterol treatment.

Cholesterol esterification in CHO-OSBP and mock-transfected cells responded in a similar manner to increasing concentrations of 25-hydroxycholesterol. Both cell lines had maximal elevation in cholesterol esterification at 2.5–5 μ g of 25-hydroxycholesterol/ml, and CHO-OSBP cells displayed a consistent 40% reduction in cholesterol esterification over the entire concentration range (Figure 2).

We next tested whether CHO-OSBP cells had altered esterification of LDL-derived cholesterol (Figure 3). Similar to results with 25-hydroxycholesterol, LDL-treated CHO-OSBP cells displayed significantly decreased cholesteryl ester synthesis at all time points compared with controls. [3 H]Oleate incorporation into triacylglycerol was also decreased in CHO-OSBP cells (Figure 3B). However, unlike 25-hydroxycholesterol, LDL treatment did not suppress [3 H]oleate incorporation into triacylglycerol in mock-transfected or CHO-OSBP cells.

ACAT activity was measured in the membrane fraction of mock-transfected and CHO-OSBP cells cultured in delipidated serum, 25-hydroxycholesterol, 25-hydroxycholesterol plus cholesterol or human LDL (Table 2). Similar to the activity measured

Figure 1 Cholesterol esterification in mock- and OSBP-transfected CHO-K1 cells

Three cell lines mock-transfected with pCMV (M1, ●; M2, ▲; M3, ■) and three transfected with the wild-type rabbit OSBP cDNA (OSBP ○; OSBP △; OSBP □) were cultured for 18 h in DMEM containing 5% delipidated FCS. (A) Immunoblot analysis of OSBP in the six mock-transfected and transfected cell lines. (B and C) The six cell lines were treated with 25-hydroxycholesterol (2.5 μ g/ml) in medium containing 5% delipidated FCS for the indicated times. Cells received 200 μ M [3 H]oleate complexed to BSA for the last 30 min of oxysterol treatment. [3 H]Oleate incorporation into cholesteryl ester (B) and triacylglycerol (C) was determined as described in the Materials and methods section. Results are the means of duplicate determinations from a representative experiment.

in intact cells, ACAT activity in membranes from CHO-OSBP cells cultured in lipoprotein-free medium was 50% of that in similarly treated control cells. ACAT activity in membranes from CHO-OSBP cells treated with oxysterol was reduced by approximately one-half, compared with a significant reduction of only 25% in membranes from LDL-treated CHO-OSBP cells. ACAT activity was also reduced in membranes from two other overexpressing lines (OSBP 7 and 18) compared with mock-transfected controls (results not shown).

Table 1 Effect of oxysterols on [3 H]oleate incorporation into cholesteryl ester, triacylglycerol and phospholipids of mock-transfected and CHO-OSBP cells

CHO-OSBP (clone 16) cells and a mock-transfected cell line (clone M3) were cultured in medium containing 5% delipidated FCS for 18 h. Cells then received oxysterol (2.5 μ g/ml) for 4 h and were labelled with 200 μ M [3 H]oleate/BSA for the final 30 min. Isotope incorporation into cholesteryl ester, triacylglycerol and total phospholipid was determined. Results are the means $\pm$ S.D. for three experiments. Abbreviations are: 25-OH, 25-hydroxycholesterol; 7-Oxo, 7-oxocholesterol; 20-OH, 20-hydroxycholesterol; 22(S)-OH, 22-(S)-hydroxycholesterol; 22(R)-OH, 22-(R)-hydroxycholesterol; 7 β -OH, 7 β -hydroxycholesterol; 19-OH, 19-hydroxycholesterol; NA, no addition. * P < 0.05, ** P < 0.025, *** P < 0.01, **** P < 0.005 compared with similarly treated mock-transfected cells.

Oxysterol addition	[3 H]oleate incorporation (pmol/min/mg protein)					
	Cholesteryl ester		Triacylglycerol		Phospholipids	
	Mock	CHO-OSBP	Mock	CHO-OSBP	Mock	CHO-OSBP
NA	16.1 $\pm$ 3.6	5.9 $\pm$ 1.3***	133.9 $\pm$ 14.2	113.5 $\pm$ 5.8	392.4 $\pm$ 24.8	417.5 $\pm$ 57.4
25-OH	72.0 $\pm$ 14.2	37.4 $\pm$ 6.4**	69.0 $\pm$ 15.6	43.8 $\pm$ 2.0	368.9 $\pm$ 57.3	372.4 $\pm$ 3.6
7-Oxo	40.3 $\pm$ 5.9	20.8 $\pm$ 2.0***	99.9 $\pm$ 26.1	77.4 $\pm$ 7.2	363.4 $\pm$ 42.0	372.4 $\pm$ 43.3
20-OH	41.0 $\pm$ 6.0	16.7 $\pm$ 3.2****	67.1 $\pm$ 14.1	39.8 $\pm$ 5.6*	412.7 $\pm$ 38.0	419.0 $\pm$ 62.2
22(S)-OH	32.6 $\pm$ 5.7	14.9 $\pm$ 2.6***	101.3 $\pm$ 16.9	58.6 $\pm$ 10.9**	386.1 $\pm$ 17.0	360.7 $\pm$ 70.2
22(R)-OH	39.9 $\pm$ 15.8	15.8 $\pm$ 9.0***	130.3 $\pm$ 30.7	108.9 $\pm$ 21.4	435.5 $\pm$ 21.0	452.8 $\pm$ 54.2
7 β -OH	22.2 $\pm$ 4.1	9.8 $\pm$ 1.9***	115.7 $\pm$ 25.6	83.8 $\pm$ 5.0	394.0 $\pm$ 62.9	385.4 $\pm$ 55.0
19-OH	27.4 $\pm$ 8.1	13.9 $\pm$ 2.0*	88.5 $\pm$ 2.7	77.3 $\pm$ 9.5	331.2 $\pm$ 27.5	381.2 $\pm$ 44.5

Figure 2 Effect of 25-hydroxycholesterol concentration on cholesterol esterification in cells overexpressing OSBP

Mock-transfected (●) and CHO-OSBP (△) cells were cultured in medium containing 5% delipidated FCS for 18 h followed by a 4 h treatment with increasing concentrations of 25-hydroxycholesterol. Cholesteryl ester synthesis was measured by incorporation of 200 μ M [3 H]oleate/BSA during the last 30 min of oxysterol treatment. Results are the means $\pm$ S.D. for three experiments. * P < 0.025, ** P < 0.001 compared with mock-transfected cells.

To test whether cholesterol availability could be limiting in ACAT assays [23,24], membranes from control and CHO-OSBP cells were preincubated with increasing amounts of cholesterol dissolved in ethanol and ACAT activity was measured (Figure 4). ACAT activity in both membrane fractions was stimulated to a similar extent by cholesterol (2.5–3-fold) and reached a maximum at 10 μ g/ml. However, activity in CHO-OSBP membranes was 40–50% of control at all cholesterol concentrations tested.

Effect of OSBP overexpression on mRNA for ACAT and sterol-regulated genes

To determine if overexpression of OSBP was decreasing ACAT activity at the transcriptional level, mRNA levels were measured and compared with control cells treated with 25-hydroxy-

Figure 3 Stimulation of cholesterol esterification by human LDL in CHO-K1 cells overexpressing OSBP

After an 18 h pretreatment in medium containing 5% delipidated FCS, mock-transfected (●) and CHO-OSBP (△) cells received the same medium with 100 μ g of human LDL/ml for the indicated times. For the final 30 min of each incubation, cells were labelled with 200 μ M [3 H]oleate/BSA and incorporation into cholesteryl ester (A) or triacylglycerols (B) was quantified. Results are the means $\pm$ S.D. for three or four experiments. * P < 0.05, ** P < 0.005, *** P < 0.001 compared with mock-transfected cells.

cholesterol (Figure 5). The expression of three sterol-regulated mRNAs was also quantified. Compared with mock-transfected cells, CHO-OSBP cells grown in delipidated serum had mRNA

Table 2 ACAT activity in membranes from mock-transfected and CHO-OSBP cells

CHO-OSBP and mock-transfected cells (Mock) were grown in medium containing 5% FCS for 18 h, followed by treatment with 25-hydroxycholesterol (25-OH, 2.5 $\mu\text{g/ml}$) for 4 h, 25-hydroxycholesterol (2.5 $\mu\text{g/ml}$)/cholesterol (25-OH/Chol, 10 $\mu\text{g/ml}$) for 4 h, human LDL (LDL, 100 $\mu\text{g/ml}$) for 8 h or no addition (NA). Total cell membranes were isolated and assayed for ACAT activity. Results are the means $\pm$ S.D. for four to nine experiments. * $P < 0.005$, ** $P < 0.001$ compared with activities for membranes from mock-transfected cells.

Treatment	ACAT activity (pmol/min per mg of protein)	
	Mock	CHO-OSBP
NA	19.3 $\pm$ 3.9	7.7 $\pm$ 1.7**
25-OH	36.2 $\pm$ 2.3	18.0 $\pm$ 4.3**
25-OH/Chol	36.5 $\pm$ 5.4	19.8 $\pm$ 4.0**
LDL	66.5 $\pm$ 8.4	50.0 $\pm$ 7.3**

Figure 4 Stimulation of ACAT activity by exogenous cholesterol

Membranes were isolated from CHO-OSBP (□) and mock-transfected (■) cells grown for 18 h in medium containing 5% delipidated FCS. Equivalent amounts of membrane protein (50 μg) was incubated with increasing amounts of cholesterol dissolved in ethanol for 30 min at 37 °C prior to initiating the assay by the addition of [^{14}C]oleoyl-CoA. All assays including controls contained 1% (v/v) ethanol. Results are means of duplicate determinations from a representative experiment.

levels for ACAT that were reduced by 50%. Treatment with 25-hydroxycholesterol for up to 4 h did not affect ACAT mRNA levels. In contrast, mRNA for HMG-CoA reductase, HMG-CoA synthase and the LDL receptor was elevated by 40–60% in CHO-OSBP cells grown in medium containing delipidated serum. Treatment of mock-transfected and CHO-OSBP cells with 25-hydroxycholesterol caused parallel suppression of mRNA levels, and by 4 h the difference in transcript levels between the two cells was < 10%. The two other cell lines overexpressing OSBP displayed a similar elevation in mRNA for sterol-regulated genes and 50–60% reduction in ACAT mRNA (results not shown).

Role of the OSBP PH domain in cholesterol regulation in overexpressing cells

PH domains are found in numerous proteins involved in intracellular signalling pathways [12] and appear to be involved in

recruiting these proteins to membranes [25,26]. To determine the role of the OSBP PH domain in the altered cholesterol regulation phenotype, two cDNAs with a complete or partial N-terminal deletion of the PH domain of OSBP were constructed, functionally characterized and overexpressed in CHO-K1 cells. Figure 6 shows the results of experiments in which wild-type and mutant OSBP cDNAs were transiently transfected in COS 7 cells and the expressed proteins assayed for *in vitro* [^3H]25-hydroxycholesterol binding. The expression of these proteins in COS 7 cells was analysed by immunoblotting of cytosol and membrane fractions (Figure 6A). The levels of expression and distribution were similar, with the exception of OSBP $\Delta\text{N-PH}$, which tended to localize to the 100 000 g membrane fraction to a greater extent. [^3H]25-Hydroxycholesterol binding analysis on cytosol from COS 7 cells transfected with the wild-type and OSBP ΔPH cDNA was similar. OSBP $\Delta\text{N-PH}$ displayed approx. 20% of wild-type activity consistent with reduced expression in the cytosol fraction (Figure 6A below). The K_d for [^3H]25-hydroxycholesterol binding was similar for wild-type OSBP and the two deletion mutants (8–15 nM).

CHO-K1 cells stably overexpressing the two PH-domain-deletion mutants were isolated and analysed for OSBP expression and cholesterol esterification. The level of expressed protein in the cytosol fraction was similar between wild-type and OSBP deletion mutants (Figure 7A). More OSBP ΔPH was detected in membranes and, unlike expression in COS 7 cells, expression of OSBP $\Delta\text{N-PH}$ in membranes from CHO-K1 cells was similar to wild-type. These stably transfected lines were then tested for cholesterol esterification activity (Figure 7B). As shown in previous Figures, CHO-OSBP cells have decreased cholesterol esterification in the presence and absence of 25-hydroxycholesterol. Cholesterol esterification in CHO-K1 cells overexpressing the PH-domain-deletion mutants did not display this defect and were similar to mock-transfected controls.

Cholesterol biosynthesis in CHO-OSBP cells and cells overexpressing the OSBP deletion mutants was measured by [$1\text{-}^{14}\text{C}$]acetate incorporation (Table 3). [^{14}C]Acetate incorporation into cholesterol of CHO-OSBP cells was increased by 80% compared with untreated mock-transfected controls, OSBP $\Delta\text{N-PH}$ - and OSBP ΔPH -transfected cells. Cholesterol synthesis in all four cell lines were suppressed to a similar level (80–90%) by growth in 25-hydroxycholesterol (2.5 $\mu\text{g/ml}$) for 8 h. [^{14}C]Acetate-labelling of lanosterol, a methylated intermediate in cholesterol synthesis, in untreated cells was not appreciably affected by overexpression of wild-type or OSBP mutants. 25-Hydroxycholesterol reduced lanosterol labelling by 60% in controls and from 80 to 50% in the transfected cell lines.

Translocation of OSBP to the Golgi apparatus appears to be an important response to oxysterol binding [11]. Given the putative role of the PH domain in membrane localization, we tested by indirect immunofluorescence whether the PH-domain-deletion mutants localized to the Golgi apparatus in response to 25-hydroxycholesterol (Figure 8). As previously reported [11], wild-type OSBP in CHO-OSBP cells converts from a punctate or diffuse pattern (Figure 8A) to prominent juxtanuclear staining, indicative of the Golgi apparatus (Figure 8B), when exposed to 25-hydroxycholesterol. Untreated cells overexpressing OSBP $\Delta\text{N-PH}$ and OSBP ΔPH also displayed a diffuse staining pattern, but with little evidence of vesicular or punctate staining (Figures 8C and 8E). The immunofluorescence staining patterns shown in Figures 8(C) and 8(E) were not altered when cells were treated with 25-hydroxycholesterol (Figures 8D and 8F). Exposure of these cells to 25-hydroxycholesterol for up to 8 h did not promote localization of PH deletion mutants of OSBP to the Golgi apparatus.

Figure 5 mRNA levels for ACAT and sterol-regulated genes in mock-transfected and OSBP-overexpressing CHO cells

CHO-OSBP and control cells were cultured in medium containing 5% delipidated FCS for 18 h prior to treatment with 25-hydroxycholesterol (2.5 μ g/ml) in the same medium for the indicated times. mRNA for four sterol-regulated genes in CHO-OSBP (□) and mock-transfected control (■) cells was quantified by S1 nuclease protection assays, normalized to expression of glyceraldehyde-3-phosphate dehydrogenase mRNA and expressed relative to untreated controls. Results are the means $\pm$ S.D. four or five separate experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.005$, n.s. (not significant) compared with mock-transfected cells.

DISCUSSION

CHO-OSBP cells displayed an altered cholesterol regulation phenotype characterized by up-regulation of cholesterol synthesis and down-regulation of cholesterol esterification. This phenotype was evident in cells that were cultured in lipoprotein-free medium in the absence of 25-hydroxycholesterol, and was characterized by a 40–60% increase in mRNA for three sterol-regulated genes, an 80% increase in [14 C]acetate incorporation into cholesterol and decreased ACAT activity and mRNA. When exposed to oxysterol, suppression of cholesterol synthesis in CHO-OSBP cells was normal, and the magnitude of the reduction for sterol-regulated mRNAs was greater than observed in controls. Thus the primary mediator for enhanced cholesterol synthesis in overexpressing cells appears to be the unoccupied form of OSBP, and there is sufficient endogenous and transfected rabbit OSBP to ensure a normal or slightly exaggerated response to oxysterol treatment.

CHO-OSBP cells displayed constitutive down-regulation of ACAT activity that could not be overcome by incubating cells in LDL or 25-hydroxycholesterol, or *in vitro* by exogenous cholesterol. ACAT activity is thought to be regulated primarily by substrate availability [23,24]. However, substrate limitation cannot explain the results as incubation of membranes from CHO-OSBP cells with saturating amounts of cholesterol did not correct the ACAT deficiency. Rather, reduced ACAT activity appears to result from a similar reduction in mRNA levels in OSBP overexpressing cells. Recently, ACAT mRNA was shown to be elevated 2-fold in the liver, but not in other tissues, of the cholesterol-fed mouse [27]. In CHO-K1 cells we found no evidence for alteration of ACAT mRNA using 25-hydroxycholesterol treatments that suppressed mRNA for LDL receptor, HMG-CoA reductase and HMG-CoA synthase.

In addition to effects on cholesterol synthesis and esterification, OSBP overexpression had pronounced, albeit inconsistent, effects on [3 H]oleate incorporation into triacylglycerol. 25-Hydroxycholesterol treatment of control and CHO-OSBP cells suppressed oleate incorporation into triacylglycerol, and overexpressing cells had triacylglycerol labelling that was usually lower than controls, regardless of the treatment. This did not appear to be due to differences in [3 H]oleate uptake or metabolism, since incorporation into total phospholipid was normal and substantially greater than either triacylglycerol or cholesteryl ester labelling. However, triacylglycerol synthesis was not consistently suppressed in CHO-OSBP cells; one clone (CHO-OSBP-18) appeared to have normal synthesis, and there was variation in response to treatment with different oxysterols. The reasons for this are unclear, but culture conditions may not have been optimal for measuring triacylglycerol metabolism or prolonged OSBP expression may enhance the triacylglycerol synthesis defect.

As a further step toward understanding how OSBP might function to regulate cholesterol metabolism in overexpressing cells, we investigated the role of the PH domain (amino acids 92–182) in conferring the altered regulation phenotype. Although differing in primary sequence, several PH domains are reported to have a common highly conserved tertiary structure that could form a surface for interaction with other factors [28–30]. PH domains have been shown to interact with the $\beta\gamma$ subunits of heterotrimeric G-proteins [31,32], protein kinase C isoforms [33–35] and phosphatidylinositols [36–40]. However, diversity in PH domain sequences may signify the existence of multiple ligands. Consistent with this and other findings regarding the role of the PH domain in membrane localization and complex-formation, deletion of the PH domain of OSBP resulted in loss of 25-hydroxycholesterol-mediated translocation to the Golgi

Figure 6 [^3H]25-Hydroxycholesterol binding by PH-domain-deletion mutants of OSBP

Wild-type and PH-domain-deletion mutants of OSBP were transiently overexpressed in COS 7 cells and membrane and soluble fractions isolated by centrifugation. (A) Immunoblot analysis of cytosol (lanes C) and membrane (lanes M) fractions from COS 7 cells. (B) [^3H]25-Hydroxycholesterol specific binding curves for cytosol from cells expressing mock (●), wild-type OSBP (Δ), OSBP ΔPH (□) and OSBP ΔN-PH (■). Specific binding was determined by subtraction of non-specific binding measured in the presence of a 200-fold excess of unlabelled 25-hydroxycholesterol. Results are means of duplicate determinations from a representative experiment.

apparatus. Even in the absence of 25-hydroxycholesterol, both deletion mutants had less staining of small punctate structures in the perinuclear region compared with the wild-type receptor. In addition, CHO-K1 cells overexpressing the two PH-domain-deletion mutants did not display increased cholesterol synthesis or decreased ACAT activity. These results suggest that correct intracellular localization of OSBP in the Golgi/vesicular compartment is necessary to induce altered cholesterol regulation. Like the ligand-binding domain of OSBP [11], the PH domain appears to play a key role in intracellular localization, perhaps targeting the protein in the vesicular/Golgi compartment.

Both of the deletion mutants used in the present study bound [^3H]25-hydroxycholesterol as effectively as the wild-type, demonstrating that the receptor is not misfolded and inactive. This finding also serves as an important control regarding the interpretation of the overexpression experiments. It is feasible that overexpression of a binding protein may non-specifically sequester a regulatory ligand that would otherwise interact with its true receptor. While both PH-domain-deletion mutants bound 25-hydroxycholesterol and were expressed in cells to a similar level as the wild-type protein, cell lines expressing these proteins do not have altered ACAT or cholesterol synthesis. If the mutant proteins were simply acting as a sink for endogenous or exogenous

Figure 7 Stimulation of cholesterol esterification by 25-hydroxycholesterol in CHO-K1 cells overexpressing PH-domain-deletion mutants and wild-type OSBP

Cells were cultured for 18 h in medium containing 5% delipidated FCS prior to addition of 25-hydroxycholesterol. (A) Expression of OSBP in the soluble and membrane fractions of mock-transfected (M3) and stably transfected CHO-K1 cell lines was assessed by immunoblotting with monoclonal 11H9. (B) Cholesterol esterification in mock-transfected controls (●), CHO-OSBP cells (Δ), CHO-OSBP ΔPH cells (□) and CHO-OSBP ΔN-PH cells (■) was measured for the indicated treatment times with 25-hydroxycholesterol (2.5 $\mu\text{g}/\text{ml}$) in medium containing 5% delipidated FCS as described in the legend to Figure 1. Results are the means $\pm$ S.D. of three experiments. Results for CHO-OSBP cells were significantly different from the three other transfected lines at 0 ($P < 0.025$) and 4 h ($P < 0.05$).

Table 3 Incorporation of [^{14}C]acetate into sterols of CHO-K1 cells expressing wild-type and PH-domain-deletion mutants of OSBP

Cells were cultured in medium B for 18 h prior to the addition of 25-hydroxycholesterol (2.5 $\mu\text{g}/\text{ml}$) or ethanol solvent in medium for 8 h. During the last 2 h of each treatment, cells were labelled with [^{14}C]acetate (7.5 mCi/ml, 55 mCi/mmol), harvested, and isotope incorporation into cholesterol and lanosterol was determined. Results are the means $\pm$ S.D. for three or four experiments. * $P < 0.05$, ** $P < 0.025$ compared with no addition (NA) or 25-hydroxycholesterol-treated mock-transfected cells.

	[^{14}C]Acetate incorporation (d.p.m./2 h per mg of protein)			
	Cholesterol		Lanosterol	
Cell line	NA	25-OH	NA	25-OH
Mock	54.5 $\pm$ 8.2	6.5 $\pm$ 3.0	100.0 $\pm$ 16.2	35.9 $\pm$ 15.2
CHO-OSBP	89.7 $\pm$ 15.0**	10.3 $\pm$ 3.6	102.0 $\pm$ 13.2	19.7 $\pm$ 8.2
OSBP ΔN-PH	48.8 $\pm$ 16.7	6.3 $\pm$ 3.9	72.0 $\pm$ 16.1	14.9 $\pm$ 2.6*
OSBP Δ6PH	50.4 $\pm$ 16.2	8.8 $\pm$ 5.9	75.3 $\pm$ 1.6	32 $\pm$ 7.7

ligand, a phenotype similar to CHO-OSBP cells would be predicted. Similarly, cholesterol esterification in CHO-OSBP cells had a similar dose-response curve for 25-hydroxycholesterol

Figure 8 Immunofluorescence localization of OSBP PH-domain-deletion mutants in CHO-K1 cells

Cells were cultured in medium containing 5% delipidated FCS for 18 h prior to treatment with 25-hydroxycholesterol (2.5 $\mu\text{g}/\text{ml}$; **B**, **D** and **F**) or ethanol solvent (**A**, **C** and **E**) in the same medium for 2 h. Indirect immunofluorescence localization of OSBP in CHO-OSBP (**A** and **B**), OSBP ΔPH (**C** and **D**) and OSBP $\Delta\text{N-PH}$ (**E** and **F**) cells was determined using monoclonal 11H9. The bar represents 10 μm .

compared with control cells (Figure 2), indicating that OSBP was not acting as a non-specific buffer for oxysterol.

In conclusion, overexpression of OSBP in CHO cells has multiple, co-ordinate effects on cholesterol synthesis and esterification that point to a key role for this receptor in mediating the effects of oxysterols or other unidentified endogenous ligands.

This work was supported by a Medical Research Council of Canada Program grant PG-11476 and a Scholarship to N.D.R. We gratefully acknowledge the excellent technical assistance of Robert Zwicker and Gladys Keddy in tissue-culture and transfection studies. We also thank Margo Storey for preparation of lipoprotein-free serum.

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