COORDINATE REGULATION OF PHOSPHATIDYLCHOLINE, SPHINGOMYELIN, CHOLESTEROL AND FATTY ACID METABOLISM IN STEROL REGULATORY DEFECTIVE CHINESE HAMSTER OVARY CELLS

By

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Dedicated To My Parents

Art and Janet Storey

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Abstract

We investigated the mechanism for covariance of phosphatidylcholine (PtdCho), sphingomyelin (SM), and cholesterol observed in various pathological and experimental conditions using three sterol regulatory defective (SRD) Chinese hamster ovary (CHO) cell lines. SRD 6 cells are cholesterol auxotrophs, SRD 2 possess constitutive cholesterol synthesis and SRD 4 cells display increased cholesterol synthesis and lack cholesterol esterification. In SRD 6 cells, [^{3}H]choline incorporation into PtdCho was decreased 50% due to of inhibition of phosphocholine conversion to CDP-choline (catalyzed by CTP:phosphocholine cytidylyltransferase, CT). In SRD 6 cells, CT mass was increased in the cytosol fraction, but membrane-bound CT activity and content were similar to controls. SRD 6 cells exhibited decreased synthesis of fatty acid, a known activator of CT. Accordingly, PtdCho synthesis in SRD 6 cells was normalized by treatment with oleate. In SRD 2 and SRD 4 cells, PtdCho synthesis was similar to CHO 7 values, but [^{3}H]glycerophosphocholine levels were increased indicating stimulated PtdCho catabolism. Results suggest that SREBP activation and resultant effects on fatty acid biosynthesis were the primary factors regulating PtdCho synthesis in the SRD 6 cells. In addition to aberrant PtdCho metabolism, basal and 25-OH cholesterol-stimulated SM synthesis were affected. In SRD 6 and wild-type CHO-K1 cells treated with cholesterol synthesis and transport inhibitors, basal SM synthesis was decreased. 25-OH cholesterol-stimulation of SM synthesis was inhibited in cholesterol-depleted SRD 6 cells, but cholesterol supplementation restored this stimulation. Regulation of SM by oxysterols and cholesterol may occur through the regulation of the oxysterol binding protein (OSBP). Conditions which inhibit oxysterol stimulation of SM also caused dephosphorylation of OSBP. In the case of cholesterol-depleted SRD 6 cells, this was accompanied by constitutive Golgi localization. Results from these studies demonstrated that cholesterol levels influenced the phosphorylation and intracellular localization of OSBP and affected its ability to alter SM synthesis in response to 25-OH cholesterol.

List of Abbreviations

25-OH	25-hydroxycholesterol
ab	antibody
ACAT	acyl-CoA:cholesterol acyltransferase
ADD	adipose determination and differentiation-dependent factor
ALLN	N-acetyl-leucyl-leucyl-norleucinal
BFA	brefeldin A
bHLH-Zip	basic-helix-loop-helix-leucine zipper
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
CE	cholesterol ester
CDP-choline	cytidine 5'-diphosphocholine
Cer	ceramide
CHO	Chinese hamster ovary
Chol	cholesterol
CoA	coenzyme A
cpm	counts per minute
CK	choline kinase
CPT	choline phosphotransferase
CT	CTP:phosphocholine cytidylyltransferase
DETAPAC	diethylenetriaminepenta-acetic acid
DMEM	Dulbecco's modified Eagles medium

DMSO	dimethylsulfoxide
dNTP	dideoxynucleotide triphosphate
dpm	disintegrations per minute
DTT	dithiothreitol
EDTA	ethylene-diaminetetra-acetic acid
EGTA	ethylene glycol-(*bis*)(β-aminoethyl ether)-N,N,N',N'-tetra-acetic acid
ER	endoplasmic reticulum
FITC	fluorescein-5-isothiocyanate
FCS	fetal calf serum
Hepes	4-(2-hydroxyethyl)-1-piperazine-N-[2-ethanesulfonic acid]
GlcCer	glucosylceramide
GST	glutathione *S* -transferase
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GroPcho	glycerophosphocholine
HMG	3-hydoxy-3-methylglutaryl
IC_{50}	inhibition concentration (causing 50% inhibition)
IUP	ideal upper phase
kDa	kilo-dalton
LB	Luria Bertain Broth
LDL	low density lipoprotein
LPDS	lipoprotein-deficient serum
lyso-PtdCho	lysophosphatidylcholine

NP	Niemann-Pick disorders
OSBP	oxysterol binding protein
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PDMP	D-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol
PEG	polyethyleneglycol
Pcho	phosphocholine
PIPES	piperazine-*N,N'*-bis[2-ethanesulfonic acid]
PM	plasma membrane
PEMT	phosphatidylethanolamine methyltransferase
PMSF	phenylmethanesulfonyl fluoride
PtdCho	phosphatidylcholine
PtdEtn	phosphatidylethanolamine
PtdIns	phosphatidylinositol
PtdSer	phosphatidylserine
PVDF	polyvinylidene difluoride
SCAP	SREBP cleavage activation protein
SDS	sodium dodecyl sulfate
SEM	standard error about the mean
SM	sphingomyelin
SPT	serine palmitoyltransferase
SRE	sterol response element
SREBP	sterol regulatory element binding protein

SRD	sterol regulatory defective
TBS	Tris-buffered saline
TCA	trichloroacetic acid
TE	Tris/EDTA buffer
TLC	thin-layer chromatography
TPCK	L-(tosylamido-2-phenyl) chloromethyl ketone
Tris	Tris (hydroxymethyl)aminomethane
v/v	volume per volume
w/v	weight per volume
U18666A	3-ß-(2-diethylaminoethoxy)androst-5-en-17-one hydrochloride

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I. Introduction

Biological membranes play a key role in cellular physiology and function. Membranes are important for compartmentalizing functions and pathways within the various organelles, providing a barrier to the external environment, regulating the transport of proteins and lipids, and cell-to-cell communication. Membranes are also the site for generation of lipid signaling molecules that affect cell growth, gene expression and cell differentiation (Lambeth and Ryu, 1996; Merrill and Sweely, 1996). The physical composition of the lipid bilayer plays an important role in regulating these events.

Cellular membranes are dynamic structures composed of lipids (phospholipids, sphingolipids and cholesterol) and proteins. The ratios of the various lipid components influence the physical characteristics of the membrane, which include packing, bilayer width, permeability and fluidity (Cullis and Hope, 1996). All of these parameters are important in protein-lipid interactions and regulation of membrane-bound enzymes. Thus, coordinate regulation of phospholipid, sphingolipid and cholesterol composition must exist either at the level of synthesis or degradation. Understanding how cholesterol metabolism affects the synthesis and degradation of phospholipids and vice versa is paramount to understanding many pathological conditions, including atherosclerosis and neurological disorders that display abnormal lipid accumulation (Kolesnick, 1991; Spence and Callahan, 1989).

The initial hypothesis of this project was that synthesis or

degradation of phospholipids, particularly SM and PtdCho, is regulated metabolically by alterations in cholesterol homeostasis. In this study, I have employed three mutant Chinese hamster ovary (CHO) cell lines that have defective sterol regulation to determine how altered cholesterol metabolism affects SM and PtdCho synthesis.

A. Cholesterol

Since its discovery by De Fourcroy in 1789, the study of cholesterol has been a large and active field (Bloch, 1992). The sterols, cholesterol in mammals, ergosterol in yeast, and sitosterol and campesterol in plants, are essential components of cellular membranes. Cholesterol is also the precursor for the synthesis of the bile acids, corticoids, sex hormones and vitamin D.

1. Structure

Cholesterol ($C_{27}H_{46}0$; Figure 1) is comprised of three (A, B, C) 6-member and one (D) 5-member carbon-rings. These ring structures have two methyl groups attached at carbons-10 and -13, and an aliphatic hydrocarbon side chain attached at carbon-17. Cholesterol contains a single double bond between carbons-5 and -6 and a hydroxyl group at carbon-3. Cholesterol in tissues and plasma is either in the free (unesterified) form, or esterified at the 3β-hydroxyl with a long-chain fatty acid.

2. Localization

The cholesterol content of membranes varies in intracellular organelles. The majority of cholesterol (60-90%) is localized in the plasma membrane (PM)

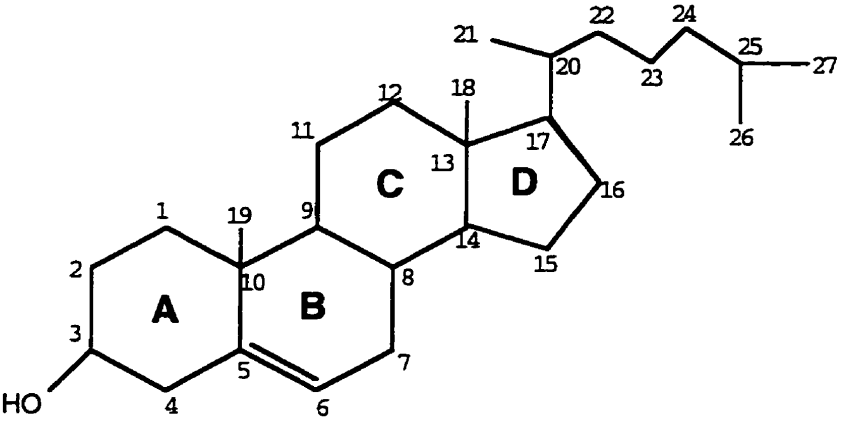

Figure 1. Structure of cholesterol

(reviewed in Liscum and Underwood, 1995; Schroeder et al., 1996; Billheimer and Reinhart, 1990). The content of cholesterol in organelles is proportional to their proximity to the PM. The general pattern of sterol concentrations is: PM > *trans* Golgi network > *trans*-Golgi > *cis/medial*-Golgi > smooth ER >> rough ER (Billheimer and Reinhart, 1990). The cholesterol contents of the peroxisomes, nucleus and the outer mitochondrial membrane are comparable to the smooth ER, and the inner mitochondrial membrane is devoid of cholesterol (Schroeder et al, 1996).

PM cholesterol is asymmetrically distributed within the bilayer, with the majority (65-90 %) localized to the inner leaflet (Schroeder et al., 1996). This cholesterol also is not evenly distributed within the leaflet, but sequestered in cholesterol-rich domains. One such domain is the caveolae, an invagination in the PM that is implicated in potocytosis and cell signaling, which requires cholesterol for its structure and function (Brown and Rose, 1992; Anderson, 1993). The Golgi apparatus also seems to have cholesterol-rich and cholesterol-poor domains which have been implicated in sorting Golgi membrane proteins from those destined for the PM (Bretscher and Munro, 1993).

3. Cholesterol biosynthetic pathway

All eukaryotes, except insects, have the ability to synthesize sterols from acetate via the isoprenoid biosynthetic pathway (Figure 2) (reviewed in Myant, 1981; Mead et al., 1986). In mammals, the liver, adrenal glands and rapidly dividing cells synthesize more cholesterol relative to other tissues because of

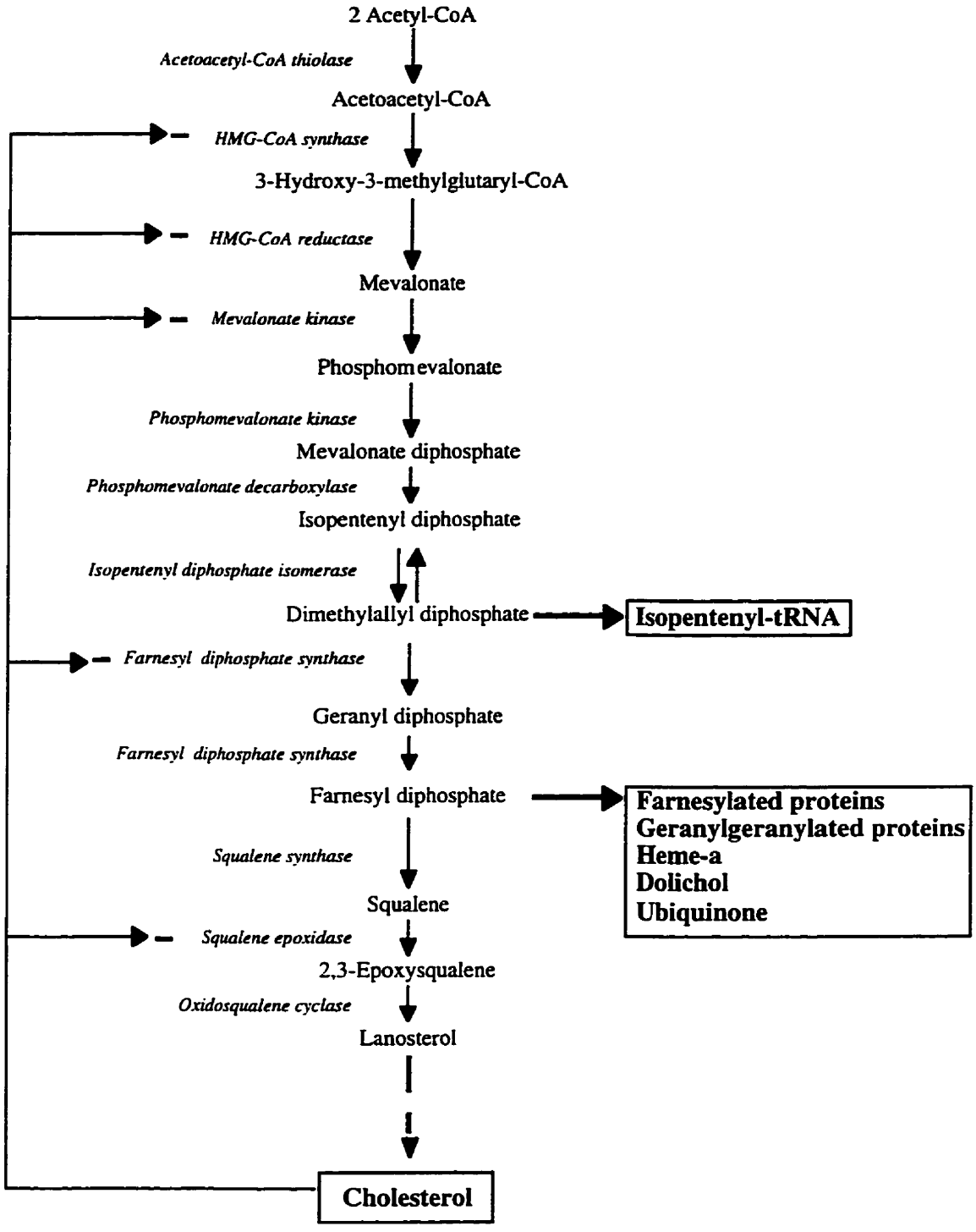

Figure 2. Isoprenoid/sterol biosynthetic pathway

requirements for lipoprotein production, hormone synthesis or cell division, respectively. The peripheral tissues appear to obtain their cholesterol from both plasma lipoproteins (Myant, 1981), and *de novo synthesis* (Dietschy et al., 1993).

The synthesis of cholesterol from acetate is catalyzed by both soluble and membrane-bound enzymes. Enzymes required for the synthesis of farnesyl diphosphate are localized to both the cytosol/ER compartment and peroxisomes (Biardi and Krisans, 1996; Krisans et al., 1994) and, with the exception of HMG-CoA reductase, these enzymes are all soluble. The remaining enzymes, except for squalene synthase, have yet to be localized, but it has been demonstrated that peroxisomes have full biosynthetic capacity (Hodge et al., 1991).

Cells which posses the ability to obtain cholesterol from plasma-derived LDL still require farnesyl diphosphate, since this isoprenoid intermediate is the precursor to many important compounds (Figure 2). There are hundreds of polyisoprenoids in nature, but the primary ones synthesized in mammalian cells are; dolichol, which is required for glycoprotein synthesis; ubiquinone and heme A, which are involved in electron transport; isopentenyl-tRNA, involved in protein synthesis; and farnesyl and geranylgeranyl diphosphate, which are added to proteins and aid in membrane association (Edwards and Davis, 1996).

4. Regulation of Cholesterol Homeostasis

The content of unesterified cholesterol within cells is highly regulated by a complex process controlling *de novo* biosynthesis, LDL uptake and cholesterol

esterification. Due to its hydrophobic nature, cholesterol accumulates within the lipid bilayer and alters physical properties of the membrane, such as membrane permeability and the activity of membrane-associated proteins (reviewed by Cullis and Hope, 1996). Only hepatic tissue can fully catabolize cholesterol and this is via the bile acid pathway (reviewed by Stravitz et al., 1993). Non-hepatic cells control cholesterol concentrations by a combination of esterification, removal of excess cholesterol from the cell to extracellular acceptors, and down-regulation of synthesis and uptake. The balance of cholesterol mass is delicate. Too little cholesterol also affects membrane structure and function. Since non-sterol products of the isoprenoid pathway are also crucial for cellular functions, the *de novo* pathway cannot be completely shut down. In the following sections regulatory mechanisms for cholesterol homeostasis will be discussed in greater detail.

a) Role of oxysterols and OSBP

Cholesterol metabolism is regulated by a feedback mechanism dependent on cellular levels of unesterified cholesterol or sterol-derivatives (Goldstein and Brown, 1990). It was first postulated by Kandutsch and Chen (1974) that oxygenated cholesterol-derivatives or oxysterols are the true regulators of cholesterol synthesis. Further studies showed that oxysterols also decreased the synthesis of the LDL receptor, thereby inhibiting cholesterol uptake, and stimulated cholesterol esterification via ACAT activation (Goldstein and Brown, 1990). Similar alterations in cholesterol synthesis and esterification resulted from increased cholesterol due to LDL-uptake, but the addition of purified

cholesterol to cultured cells had no effect (Kandutsch et al., 1978; Krieger et al., 1978; Kandutsch and Taylor, 1985). Besides altering cholesterol levels, oxysterols have other diverse biological activities including cytotoxicity, atherogenicity and inhibition of DNA synthesis in cultured cells (Schroepfer, 1981; Smith, 1981; Parish et al., 1986; Gibbons, 1983; Defay et al., 1982). It is unclear if these effects are related to oxysterol induced inhibition of cholesterol synthesis and uptake via the LDL receptor.

Different oxysterols do not have the same potency for suppression of cholesterol synthesis (reviewed in Parish et al., 1995), 25-hydroxycholesterol and 27-hydroxycholesterol are the most potent (Taylor et al., 1984; Saucier et al., 1985). The activity of oxysterols appeared to be dependent on the distance between the C-3 hydroxyl and second oxygen function, as well as an intact side-chain. These results imply that the function of oxysterols requires a structure-related interaction with a receptor. The oxysterols are more soluble than cholesterol and move to the cell interior much more rapidly (Lange et al., 1995: Morel et al., 1996), which could explain their relative potency compared to cholesterol. This property is important since regulation of cholesterol metabolism occurs primarily in the ER (Brown and Goldstein, 1997; Chang et al., 1997).

Oxysterols must naturally occur within cells for their roles in cholesterol homeostasis regulation to be physiologically relevant. 27-Hydroxycholesterol is an intermediate of bile acid biosynthesis and the conversion of cholesterol to steroid hormones produces 20- and 22-hydroxycholesterol (reviewed in Smith,

1996). Oxysterols may also be derived from plasma lipoproteins by oxidation processes (Hessler et al., 1983; Hughes et al., 1994; Colles et al., 1996). It has been shown that normal human fibroblasts produce 27-hydroxycholesterol from LDL cholesterol. This could be a mechanism for removing cholesterol from peripheral tissues (Axelson and Larsson, 1995; Axelson and Larsson, 1996). Endogenous 25-hydroxycholesterol has been isolated from cultured fibroblast (Saucier et al., 1985), but the enzymes involved in its production remain unknown.

The precise mechanism involved in oxysterol regulation of cholesterol metabolism have not been elucidated. The regulatory proteins for cholesterol synthesis and esterification are located in the ER, but the majority of cholesterol resides within the PM. Therefore it is possible that a signaling pathway involving a receptor for oxysterols regulates cholesterol movement between the PM and ER. A candidate for this proposed receptor is the oxysterol-binding protein (OSBP), which has high-affinity for oxysterols and is found in low-abundance within the cytosol (Taylor et al., 1984). The affinity of OSBP for oxysterols is proportional to their ability to inhibit HMG-CoA reductase and cholesterol synthesis (Taylor et al., 1984). Rabbit OSBP is a 809 amino acid protein that migrates as a doublet of 96 and 101 kDa on SDS-PAGE, and exists as a homodimer under native conditions (Dawson et al., 1989a). Previous work in our lab showed that this difference in molecular weight was due to phosphorylation; hyperphosphorylated OSBP migrated at 101 kDa and the dephosphorylated form at 96 kDa (Ridgway et al., 1997). Phosphorylation of

OSBP occurred at multiple serine residues (5 major phosphopeptides as shown by tryptic mapping) and was sensitive to brefeldin A, an agent that disrupts the Golgi apparatus. Staurosporine, a broad spectrum kinase inhibitor, and okadaic acid, a protein phosphatase 1 and 2A inhibitor, were the only other agents that affected OSBP phosphorylation. Interestingly, 25-hydroxycholesterol did not affect OSBP phosphorylation, and OSBP phosphorylation status did not affect *in vitro* 25-hydroxycholesterol binding (Ridgway et al., 1997).

A unique characteristic of OSBP that strengthens its hypothetical role in cholesterol trafficking is its 25-hydroxycholesterol-dependent translocation from a predominately cytosolic or vesicle-like compartment to the Golgi apparatus (Ridgway et al., 1992). Alterations in either OSBP expression or Golgi function affected regulation of cholesterol metabolism: CHO-K1 cells treated with brefeldin A, which disrupts the Golgi apparatus, were no longer responsive to transcriptional suppression by 25-hydroxycholesterol (Ridgway and Lagace, 1995), and; cells overexpressing OSBP displayed transcriptional activation of sterol-regulated genes, increased cholesterol synthesis and constitutive down-regulation of ACAT activity (Lagace et al., 1997). These results support the hypothesis that OSBP may be involved in the trafficking of cholesterol or oxysterols thereby regulating cholesterol metabolism.

b) Intracellular lipoprotein metabolism and cholesterol regulation

Since the *de novo* biosynthetic pathway for cholesterol is complex and energetically expensive, peripheral tissues also have the ability to obtain

cholesterol from the plasma. For this to occur the cholesterol must be present in a form that is easily obtained and the cell must have a regulated mechanism for ingestion. The major source of exogenous cholesterol for non-hepatic cells is low density lipoprotein (LDL) (Goldstein et al., 1985). If cells relied strictly on the diffusion of free cholesterol from the plasma, delivery would be inefficient since the cholesterol concentration gradient favors export and diffusion is not regulated (Schroeder et al., 1996). The uptake of LDL by cells involves a receptor-mediated endocytotic event, followed by transport to the lysosomes. In the lysosomes the LDL particles are degraded to amino acids, free cholesterol, and free fatty acids. The unesterified cholesterol can be released from the lysosome and transported to PM or the ER, where it is either re-esterified or mixed with the endogenous cholesterol pool (Goldstein et al., 1985). Regulation of LDL uptake occurs by altering the rate of transcriptional initiation of the LDL receptor gene (Goldstein and Brown, 1990).

c) Sterol-regulated gene expression

The primary mechanism for regulating cholesterol metabolism is the coordinate transcriptional regulation of multiple genes involved in cholesterol synthesis and uptake. Many studies have shown that increased cholesterol-uptake, or addition of exogenous oxysterols, decreased LDL receptor, HMG-CoA reductase, HMG-CoA synthase, farnesyl diphosphate synthase and squalene synthase transcription (Goldstein and Brown, 1990; Guan et al., 1995; Spear et al., 1992). These alterations in transcription result in inhibition of cholesterol uptake via the LDL receptor, and *de novo* synthesis via the

isoprenoid pathway. Under sterol-deplete conditions, increased cholesterol synthesis and uptake occurs by transcriptional activation of these coordinately regulated genes.

HMG-CoA reductase, the rate-limiting step for the isoprenoid pathway, has multiple effectors of its regulation. Regulation of transcription, besides being sterol-regulated, was shown to be tissue specific and affected by genetic and dietary factors (Hwa et al., 1992). The HMG-CoA reductase gene has multiple initiation sites and alternate sites of polyadenylation at the 3'end of the gene that produce several size classes of HMG-CoA reductase mRNA (Reynolds et al., 1985; Ramharack et al., 1990; Luskey, 1987; Reynolds et al., 1984). The roles of the multiple mRNA classes in HMG-CoA reductase regulation have not been determined.

Regulation of gene expression by cholesterol has been difficult to elucidate due to the localization of the factors involved. The majority of cholesterol resides within the PM, but transcription is regulated within the nucleus. Thus the cell must have a mechanism to activate gene transcription remotely. Early studies with the HMG-CoA reductase, LDL receptor and HMG-CoA synthase genes showed that each contained a DNA sequence responsible for sterol regulation. The 5' flanking regions of the genes for HMG-CoA synthase and the LDL-receptor contain one to three copies of a 10 bp nucleotide sequence with the consensus sequence 5'-ATCACCCCAC-3'. This sequence is a conditional positive element that enhances LDL receptor and HMG-CoA synthase gene transcription in the absence, but not presence, of sterols. This element was

termed the sterol regulatory element-1 or SRE-1 (Briggs et al., 1993; Smith et al., 1988; Yokoyama et al., 1993; Hua et al., 1993). In 1993, a sterol regulatory element binding protein (SREBP) was purified from nuclear extracts of cultured human HeLa cells on the basis of its ability to bind the SRE-1 sequence (Briggs et al., 1993; Wang et al., 1993). Since its initial identification two members of the SREBP family have been identified by cDNA cloning (Yokoyama et al., 1993; Hua et al., 1993).

The SREBPs are a novel family of membrane-bound transcription factors which require sequential proteolytic cleavage events to activate the transcription factor portion of the protein (reviewed in Brown and Goldstein, 1997). The SREBPs have a tripartite structure containing a N-terminal transcription factor domain, a middle hydrophobic region consisting of two transmembrane segments and a C-terminal regulatory domain. The SREBPs in their full length form are localized in the ER/nuclear membrane with both the N-terminal and C-terminal domains facing the cytosol (Hua et al., 1995a; Duncan et al., 1997). Two proteolytic cleavage events release the transcription factor domain from the transmembrane domains, thus allowing entry into the nucleus and binding to the SREs.

To date, two SREBPs have been cloned, SREBP-1 and SREBP-2. SREBP-1 has two isoforms, 1-a and 1-c (also known as adipose determination and differentiation-dependent factor 1, ADD-1), which are derived by alternate transcription start sites encoding different first exons, spliced to a common second exon (Yokoyama et al., 1993; Hua et al., 1995b; Shimomura et al.,

1997; Tontonoz et al, 1993). The mRNAs for SREBP1-a, and 1-c in humans, but not mice, undergo alternative splicing at the 3'ends to produce proteins that differ in the last 113 amino acids (Yokoyama et al., 1993; Hua et al., 1995b; Shimomura et al., 1997). SREBP-2 is encoded by a separate gene and seems to produce only one transcript (Hua et al., 1993; Miserez et al., 1997). SREBP1-a is a protein of 1147 amino acids, and SREBP-2 has 1141 amino acids. The overall amino acid sequence identity between SREBP-2 and SREBP-1a is 47% (Hua et al., 1993).

The SREBPs are members of the basic-helix-loop-helix-leucine zipper (bHLH-Zip) transcription factor family. The N-terminal region (approximately 480 amino acids) begins with an acidic domain, which has 33% identity between SREBP-1a and SREBP-2. This domain confers the ability to activate transcription, and when deleted SREBPs bind to DNA and transcription is inhibited (Hua et al., 1993; Sato et al., 1994). The acidic region of SREBP-1c (24 amino acids) is shorter than that in SREBP-1a (42 amino acids) or SREBP-2 (48 amino acids) and is a weaker transcriptional activator (Shimano et al., 1997). The acidic region is followed by a sequence rich in proline, serine, glycine and glutamine that is variable between SREBP-1 and SREBP-2. The next domain of the transcription factor portion is the classic bHLH-Zip sequence that displays 71% identity between the SREBPs (Hua et al., 1993). The basic region of the bHLH-Zip binds to specific nucleotide sequences within promoter elements, and the adjacent HLH-Zip regions mediate homo- or heterodimerization. Other bHLH-Zip proteins recognize a E-box motif, an

inverted repeat of the sequence CANNTG (Murre and Baltimore, 1992). SREBPs recognize the E-box motif *in vitro*, but *in vivo* they bind to the SRE sequences that contain a direct repeat of 5'-PyCAPy-3' (Magana and Osborne, 1996). The reason SREBPs recognize a direct, rather than indirect, repeat is due to a tyrosine residue that replaces a conserved arginine residue found in all other bHLH-Zip proteins. The SREBPs seem to bind DNA as homodimers.

The activation of the SREBPs requires a two step proteolytic cleavage cascade (Wang et al., 1994; Hua et al., 1996b; Sakai et al., 1996). In sterol-depleted cells the cascade is initiated by a protease that clips the SREBP at a site in the middle of the lumenal loop (Duncan et al., 1997). When either sterol or oxysterols are present the cleavage at site-1 is down-regulated. After cleavage at site-1, both fragments of the SREBP are attached to the membrane, and a second cleavage is necessary to release the transcription factor domain. This second cleavage site is within the first transmembrane domain (Hua et al., 1996b; Sakai et al., 1996). Cleavage at this second site does not seem to be directly regulated by sterols, but does not occur until site-1 proteolysis has taken place. Thus, in sterol replete cells the SREBPs remains membrane bound and is unable to access the nucleus.

The proteases involved in processing the SREBPs have not been cloned or identified, but their intracellular sites are known. Full-length SREBP precursors are located in the ER/nucleus, and the full length SREBP does not seem to be transported to the Golgi complex (Sato et al., 1994; Hua et al., 1995a; Duncan et al., 1997). Thus the first protease must reside within lumen of the ER or the

contiguous nuclear envelope. The second protease appears to reside either in the ER or in the Golgi (Brown and Goldstein, 1997). The fate of the C-terminal domain is not known.

The SREBP cleavage activation protein or SCAP was recently cloned from 25-RA cells, a mutant line that fails to suppress cleavage of SREBPs in the presence of sterols (Hua et al., 1996a). In these cells, mutant SCAP stimulated cleavage of SREBPs regardless of sterol content. SCAP is a membrane-bound protein with two domains: a N-terminal domain (730 amino acid) with eight membrane spanning sequences separated by short hydrophilic loops (Hua et al., 1996a), and; a C-terminal domain (546 amino acids) containing four WD repeats, which are important in protein-protein interactions. The C-terminus of SCAP, like that of the SREBPs is located on the cytosolic side of the ER/nuclear envelope where the protein is embedded. Recently it has been shown that the C-terminal domains of these two proteins form a complex (Sakai et al., 1997). The N-terminal domain sequence of SCAP resembles a corresponding transmembrane domain of HMG-CoA reductase, which imparts sterol-dependent proteolysis of this enzyme (Hua et al., 1996a). SCAP itself did not appear to be a protease and it failed to cleave SREBP-1 in a co-translation assay (Sakai et al., 1997). Its role in stimulating cleavage is most likely to form a complex with SREBPs so that a third protein, a protease, can associate with and cleave SREBP. The presence of sterols did not alter the SREBP-SCAP complex, but may inhibit the association of the putative protease.

The genes involved in fatty acid synthesis and uptake, acetyl CoA carboxylase, fatty acid synthase, stearoyl CoA desaturase-1 and lipoprotein lipase are also regulated by the SREBPs (Tontonoz et al., 1993; Kim and Spiegelman, 1996; Lopez et al., 1996; Magana and Osborne, 1996; Shimano et al., 1996). Transfection studies with the promoter regions of fatty acid synthase and acetyl CoA carboxylase demonstrated that their respective SRE sequences were bound by the SREBPs and transcription was activated (Tontonoz et al., 1993).

The differences in potency of the SREBPs has been demonstrated in both transfection and transgenic studies. Transfection studies showed that SREBP-1a and SREBP-1c have the same ability to stimulate transcription of the sterol-regulated genes. However, when present in physiological concentrations SREBP-1a was 10-times more potent than SREBP-1c for SRE-1 containing promoters (Shimano et al., 1997). In transgenic mice, SREBP-1c stimulated fatty acid biosynthesis, but had very little effect on cholesterol synthesis. SREBP-2 increased gene expression of the cholesterol biosynthetic genes and LDL receptor, but not the fatty acid biosynthetic genes (Shimano et al., 1997).

The SREBPs require other transcription factors to activate gene expression. Activation of expression for the LDL receptor required both a functional SREBP and Sp1 (Sanchez et al., 1995), whereas activation of transcription of HMG-CoA synthase and farnesyl diphosphate synthase required NF-Y (Jackson et al., 1995). Thus, not only the type of SRE present but the availability of other

transcription factors regulates the gene expression of the sterol-regulated genes.

The second mechanism for control of gene expression by the SREBPs is the differential expression of the various SREBPs. In cultured cells, expression of SREBP-1a exceeded SREBP-1c by 2:1 and SREBP-2 was expressed at high levels (Shimomura et al., 1997). In hamsters fed a low-fat diet the mature form of SREBP-1 was present in the liver, but with relatively little SREBP-2 (Sheng et al., 1995). When animals were fed lovastatin and a bile acid binding resin, the amount of total SREBP-2 increased along with its proteolytic processing, and the processing of SREBP-1 decreased. Thus, in sterol-depleted liver nuclei more SREBP-2 than SREBP-1 was present (Sheng et al., 1995). If hamster liver has the same ratio of SREBP-1c to -1a found in mice and humans (9-fold) (Shimomura et al., 1997), than cholesterol depletion specifically inhibits the processing of SREBP-1c. Mouse adipose tissue also had excess SREBP-1c compared to 1a. By varying the ratio of SREBP-2 to SREBP-1c the balance of basal versus stimulated cholesterol synthesis can be altered. Cultured cells have a constant need for cholesterol to support membrane synthesis and a limited supply of LDL from the added serum, thus a higher potential for cholesterol synthesis is provided by expressing both SREBP-1a and SREBP-2.

d) Post-transcriptional regulation of HMG-CoA reductase

The regulation of HMG-CoA reductase is more complex than the other cholesterol biosynthetic genes. Besides transcriptional control, HMG-CoA reductase activity is regulated by alterations in mRNA translation and stability,

protein stability and phosphorylation (Dawson et al., 1991; Ness, 1983; Rosser et al., 1989; Myant, 1990). Treatment with the pharmacological agents lovastatin and compactin, which have structural similarities to HMG-CoA, competitively inhibited HMG-CoA reductase. Chronic drug treatments resulted in increased HMG-CoA reductase mRNA and protein levels, and stimulated sterol-regulated gene expression (Alberts et al., 1980; Goldstein and Brown, 1990).

Modification of HMG-CoA reductase protein stability provides a rapid method for down-regulating mevalonate synthesis. The half-life of HMG-CoA reductase was shown to be normally 2 h. Enzymatic inhibition by lovastatin increased the half-life to 11 h, but other conditions that inhibit cholesterol synthesis, such as high mevalonic acid concentrations and the presence of oxysterols, decreased the half-life for HMG-CoA reductase to 40 min (Edwards et al. 1983; Goldstein and Brown, 1990). This selective degradation of HMG-CoA reductase also occurred in the presence of farnesyl diphosphate, and farnesol, but not other isoprenoids (Correll et al., 1994). The exact mechanism for regulating protein stability has yet to be elucidated, but the second transmembrane region of the protein was required (Kumagai et al., 1995).

HMG-CoA reductase is also regulated by a phosphorylation cycle, which provides rapid and reversible regulation. HMG-CoA reductase is inactivated by AMP-activated protein kinase mediated phosphorylation. This kinase also phosphorylates and inactivates acetyl-CoA carboxylase, a key enzyme in fatty acid biosynthesis (Clarke and Hardie, 1990). Phosphorylation of HMG-CoA reductase provides a mechanism by which cholesterol and isoprenoid

biosynthesis can be regulated by ATP/AMP ratios and therefore the energetic state of the cell (Corton et al., 1994; Gillespie and Hardie, 1992; Sato et al., 1993).

e) ACAT and cholesterol homeostasis

Acyl-coenzyme A:cholesterol acyltransferase or ACAT, is the enzyme that catalyzes the addition of a long-chain fatty acid from fatty acyl-CoAs to the 3-β-hydroxyl of cholesterol (reviewed in Chang et al., 1997). The non-polar cholesteryl esters associate to form lipid droplets and provide a non-cytotoxic storage form for excess cholesterol. This cholesterol can be easily mobilized through the action of a cholesterol esterase (Klansek et al., 1996; Brown et al., 1980).

ACAT is an integral membrane enzyme that is localized in the rough ER (Reinhart et al., 1987; Doolittle and Chang, 1982a). The protein is hydrophobic with at least two transmembrane regions, and contains a variation of the leucine zipper domain near the N-terminal region of the protein suggesting the possibility of a protein-protein interaction. Sequence analysis indicated that besides acyltransferase activity, ACAT may also contain fatty acid ligase activity (Chang et al., 1993).

Since ACAT activity is required for the storage of excess cellular cholesterol, the enzyme is primarily regulated by substrate (cholesterol and the oxysterols) availability (reviewed Chang et al., 1997). The rate of cholesteryl ester synthesis in intact cells was activated when either LDL or oxysterols are added to the growth medium. This response was stimulated by cyclohexamide (Chang et al.,

1986; Chang and Chang, 1986; Tabas and Boykow, 1987; Schissel et al., 1995). Sterol regulation of ACAT did not generally occur at the level of either gene transcription or protein translation. Sterol activation of ACAT was also demonstrated *in vitro* with either whole cell extracts or microsomal fractions (Harte et al., 1993; Harte et al., 1995). When ACAT from cells grown in the presence or absence of sterols was assayed in reconstituted cholesterol/PtdCho vesicles there was no difference in activity (Cadigan and Chang, 1988; Doolittle and Chang, 1982a and b). This indicated that observed alterations in microsomal activity were the results of changes in lipid environment or substrate availability.

The mechanism by which cholesterol and oxysterols activate ACAT is not clear. It is possible that ACAT is an allosteric enzyme and sterol binding induces conformational changes that activate the enzyme (Chang et al., 1995). ACAT activity was shown to be influenced by the phospholipid composition of membranes (Mathur et al., 1983), which indicated that activation may also involve lipid-protein interactions.

The activation of ACAT by ER cholesterol is sigmoidal meaning cholesterol mass must reach a threshold level before enzyme activation (Cadigan and Chang, 1988; Doolittle and Chang, 1982a; Cheng et al., 1995). This could be important since the other key sterol-regulated proteins, HMG-CoA reductase and SREBPs, also reside in the ER (Goldstein and Brown, 1990; Brown and Goldstein, 1997). If the ER cholesterol pool is the regulatory pool that suppresses the sterol regulated proteins, then ACAT activity could potentially

inactivate this regulation. This was demonstrated by the ability of ACAT inhibition to enhances LDL receptor and HMG-CoA reductase down-regulation (Tabas et al., 1986). However, it has been shown that the concentration of LDL required to cause half-maximal activation of cholesterol esterification is 5- to 10-times greater than that required for inhibition of cholesterol synthesis and LDL receptor activity (Brown and Goldstein, 1986). This would ensure that ACAT does not inactivate other sterol responses. The threshold activation of ACAT by its substrate is also important to ensure that newly synthesized cholesterol produced in the ER (Reinhart et al. 1987) is not esterified before it can be transported to the PM.

5. Cholesterol trafficking

Intracellular cholesterol has several possible fates; incorporation into membranes, efflux to extracellular acceptors and conversion into cholesterol esters or other cell specific metabolites, such as bile acids or steroid hormones. For any of these events to occur, *de novo* synthesized or LDL-derived cholesterol must be transported from the site of synthesis (ER) or release (lysosomes), respectively. The pathways for intracellular cholesterol transport have not been fully elucidated but seem to involve a combination of aqueous diffusion, vesicle-mediated transport and protein sterol-carriers (Liscum and Underwood, 1995; Schroeder et al., 1996; Billheimer and Reinhart, 1990).

Transport of newly synthesized cholesterol from the ER is a rapid process shown to involve lipid-rich vesicles and require energy (Lange, 1991; Lange et al 1991; Urbani and Simoni, 1990). Treatment with agents that effect the

cytoskeletal network, lysosomal function, protein synthesis and Golgi apparatus did not affect cholesterol trafficking from the ER (Urbani and Simoni, 1990; Kaplan and Simoni, 1985).

Exogenous cholesterol obtained from internalized LDL was released from the lysosome to mix with the endogenous cholesterol pool. This cholesterol appeared in both the PM and ER, but it was recently shown that transport to the ER occurs via the PM (Neufeld et al., 1996; Lange et al., 1997). The actual mechanism of transport is not known, however, the disruption of lysosomal function by inhibition of the H^+-ATPase inhibited cholesterol movement to the PM (Furuchi, et al., 1993). Drugs that induced the NP Type-II phenotype, progesterone and the hydrophobic amines imipramine and U18666A, also caused cholesterol accumulation in the lysosome (Liscum and Faust, 1989; Liscum and Collins, 1991; Rodriguez-Lafrasse et al., 1990; Roff et al, 1991; Butler et al., 1992; Lange et al., 1997). The transport of cholesterol from the lysosome to the PM was not energy-dependent (Brasaemle and Attie, 1990; Johnson et al., 1990; Liscum, 1990), and was not affected by cytoskelatal poisons or Golgi disruption (Liscum, 1990). Transport could involve vesicles or carrier proteins such sterol carrier protein 2 (Schroeder et al., 1996).

Cholesterol also must be transported from the PM to the interior of the cell, mainly to the ER, where it can be esterified and down-regulate SREBP proteolysis. In some cells, cholesterol must also be transported to the peroxisomes or mitochondria for bile acid and steroid hormone synthesis. The flux of cholesterol from the PM to the ER was blocked by progesterone,

U18666A and imipramine but not by sphinganine or stearylamine (Harmala et al., 1994; Lange, 1994; Harmala et al., 1993). Studies using LDL-cholesterol demonstrated that the intermediate filament network of the cell is important for transport of cholesterol to the ER (Sarria et al., 1992). Since the PM is the first destination for LDL-derived cholesterol, this filament network most likely plays a key role in transfer from the PM to the interior. Transport of cholesterol to the ER was also shown to be energy-dependent and inhibited by low potassium and PtdIns 3-kinase inhibitors which block intracellular membrane vesicle trafficking (Skiba et al., 1996).

Cholesterol trafficking within the cell plays a role in cholesterol homeostasis in two ways. First, the actual location of the cholesterol within the cell determines its ability to regulate cholesterol metabolism. The sterol sensing and regulatory proteins are located within the interior of the cell, mainly within the ER. By controlling the flux of cholesterol into and out of the ER pool, sterol regulation can be controlled. Increasing the flux of cholesterol to the ER will expand the regulatory pool, activate ACAT and inhibit SREBP processing. Similarly, increasing the transfer of cholesterol from the interior to the PM will decrease the regulatory pool at the ER. Secondly, cholesterol trafficking can affect cholesterol homeostasis via the reverse cholesterol transport pathway. In this process, cholesterol from the exterior leaflet of the PM is transferred to HDL particles (Glomset, 1968; Johnson et al 1991; Fielding and Fielding, 1995). This provides peripheral tissues a mechanism to remove excess cholesterol. The key regulatory point of the reverse cholesterol transport appeared to be the

amount of cholesterol present in the donor domains of the PM (Mendez, 1995; Yancey et al., 1996), thus increased cholesterol flux from the interior of the cell to donor domains in the PM, stimulated cholesterol removal from the cell.

B. Sphingomyelin

Sphingomyelin is unique in that it falls into two of the major classes of lipid. It is considered a phospholipid due to its phosphocholine headgroup, and a sphingolipid due to its ceramide backbone. Sphingolipids are nearly ubiquitous constituents of membranes in animals, plants, fungi, yeast and some prokaryotic organisms and viruses, and have roles in membrane structure, regulation of cell-surface receptors, cell-cell communication, interactions with the extracellular matrix and functions in the immune system. Sphingomyelin, besides playing a key structural role within the PM, has a turn-over cycle that generates ceramide, an important signaling molecule in cell growth, differentiation and apoptosis (reviewed in Bell et al., 1993a and b).

1. Structure

The backbone of sphingolipids is sphingosine or sphinganine (*D*-erythro-2-amino-*trans*-4-octadecene-1,3-diol; Figure 3), an 18-carbon molecule with a 4,5-trans double bond, an amino group and two hydroxyls at positions 1 and 3. In sphingomyelin, the sphingosine is *N*-acylated with a long-chain fatty acid to produce the ceramide moiety and the phosphocholine head group is located at the 1 position.

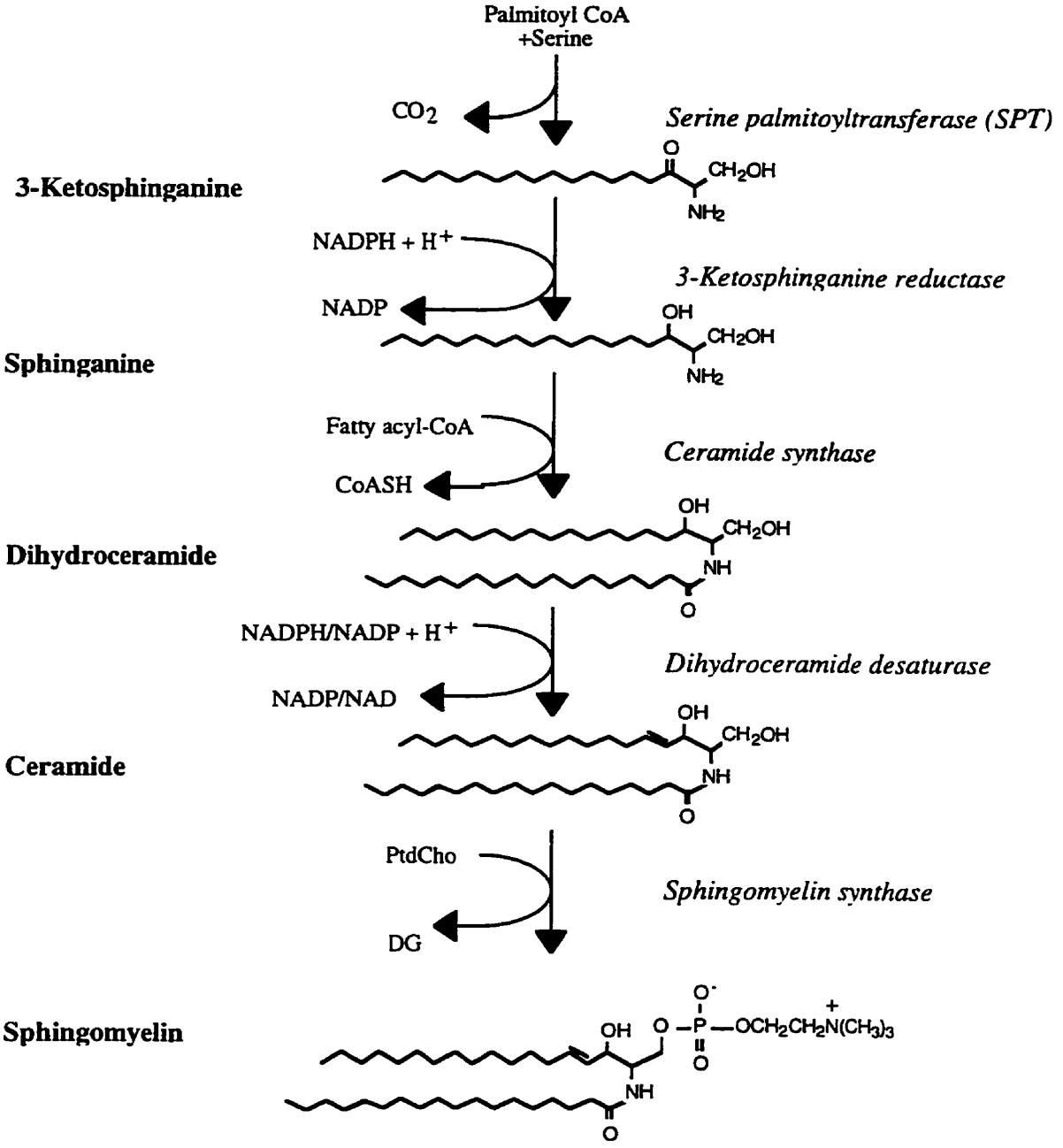

Figure 3. Sphingomyelin biosynthetic pathway

2. Biosynthetic pathway

The SM biosynthetic pathway (Figure 3) starts with the formation of ceramide and occurs at the cytoplasmic leaflet of the ER (Bell et al., 1993b). Serine palmitoyltransferase (SPT), the rate-limiting enzyme, catalyzes the condensation of palmitoyl-CoA and *L*-serine to produce 3-ketosphinganine. SPT is highly selective for palmitoyl-CoA, requires pyridoxal phosphate for activity, and is regulated by the availability of its substrates and free sphingoid bases (Merrill and Jones, 1990; Mandon et al., 1991).

The next step is the reduction of 3-ketosphinganine to sphinganine, catalyzed by 3-ketosphinganine reductase, using NADPH as a co-factor. Dihydroceramide is then formed by the *N*-acylation of sphinganine by ceramide synthase. The final step in ceramide synthesis is the insertion of the 4,5-*trans* double bond by a NADPH/NADH dependent dihydroceramide desaturase in the ER (Michel et al., 1997).

Sphingomyelin is synthesized by the transfer of phosphorylcholine from phosphatidylcholine to ceramide by the enzyme sphingomyelin synthase. In the liver, 90% of this activity was found in the *cis* and *medial* Golgi apparatus, with the remainder in the PM (Bell et al., 1993b). The PM activity appeared to be important in the SM turnover cycle and may not have a role in *de novo* synthesis (Kallen et al., 1994).

For SM synthesis to occur, ceramide must be transported from the ER to the Golgi (or PM), and transferred or "flipped" into the lumenal leaflet of the membrane for access to the enzyme (van Echten and Sandhoff, 1993).

Glucosyltransferase resides in the cytosolic leaflet of the *cis/trans* Golgi apparatus (van Echten and Sandhoff, 1993). Subsequent steps in glycosphingolipid synthesis require transbilayer movement of glucosylceramide (GlcCer) into the Golgi lumen, where the active sites of other transferases reside.

3. Regulation of sphingomyelin synthesis

SM synthesis appears to be regulated on three different levels. First the rate-limiting enzyme SPT was inhibited by high concentrations of sphingoid bases. SPT activity is also inhibited by β-haloalanines, *L*-cycloserine and a number of compounds isolated from microorganisms including sphingofungins, lipozamycins and myriocin, by a time-dependent, irreversible mechanism (Zweerink, et al., 1992; Medlock and Merrill, 1988; Miyake et al., 1995; Sundaram and Lev, 1984; Mandela et al., 1994).

SM synthesis is also regulated at ceramide synthase by the fumonisin compounds produced by strains of *Fusarium moniliforme*. The fumonisins are structurally similar to sphinganine and are competitive inhibitors of this enzyme (Merrill et al., 1993a). Treatment of cultured cells and animal models with fumonisin B_1 resulted in inhibition of sphingolipid biosynthesis and increased the levels of sphinganine, but not sphingosine (Wang et al., 1991; Merrill et al., 1993b; and Yoo et al, 1992). Related compounds, such as alternaria toxin and the structurally unrelated compound australifungi, also inhibit SPT activity. (Bell et al., 1993b).

The final level of regulation for SM synthesis is the conversion of ceramide to sphingomyelin. This step is competitively inhibited by treatment with high concentrations of PDMP (D-*threo*-1-phenyl-2-decanoylamino-3-morpholino-1-propanol), a structural analogue of ceramide (Abe et al., 1995). SM synthesis is also affected by alterations in intracellular transport. Treatment of cells with brefeldin A (BFA), a fungal toxin which promotes retrograde transport of Golgi components to the ER, caused a stimulation of SM synthesis in rat hepatocytes and BHK cells (Hatch and Vance, 1992; Kallen et al., 1993). In both these studies, treatment with forskolin, a compound which inhibits the BFA-induced collapse of the Golgi, partially blocked the activation of SM synthesis. Since SM synthase resides in the Golgi and ceramide is synthesized in the ER, BFA treatment activated SM synthesis by removing the requirement for ceramide transport. Studies from our laboratory have shown that the conversion of ceramide to SM was also increased with 25-hydroxycholesterol treatment. This effect may also result from alterations in ceramide transport (Ridgway, 1995b).

4. Cellular localization

Like cholesterol, SM has a disproportional distribution within the cell. Mass measurements have shown that up to 90% of the SM is in the PM (Barenholz and Thompson, 1980; White, 1973; Rouser et al., 1968), predominately on the external leaflet of the bilayer. The interior membranes of the cells have little (ER/Golgi) to no (inner and outer mitochondria) SM.

5. Catabolism

SM is removed from the PM by membrane internalization followed by recycling or degradation, or hydrolysis to form bioactive products that participate in cell signaling (Bell et al., 1993b). SM is internalized in endocytic vesicles and is recycled back to the PM or transported to lysosomes. In the lysosomes, it is degraded to ceramide and phosphocholine by acidic sphingomyelinase (Ferlinz et al., 1994). Neutral or alkaline sphingomyelinases hydrolyze SM at the PM or intracellular membranes, and are involved in the production of ceramide for signal transduction pathways (Bell et al., 1993a and b).

The ceramide released by sphingomyelinases may be converted to sphingosine by ceramidase (Bell et al., 1993b). Sphingosine may subsequently undergo re-acylation or phosphorylation (Bell et al., 1993b). Sphingosine-1-phosphate can be cleaved to produce ethanolamine phosphate and *trans*-2-hexadecanal (Bell et al., 1993b), which are used for PtdEtn synthesis (Smith et al., 1995; Badiani et al., 1996) or fatty acid oxidation (Bell et al., 1993b), respectively. Under certain conditions, degradation of sphingoid bases accounted for up to one third of the headgroup in PtdEtn (Smith et al., 1995).

C. Phosphatidylcholine

Phosphatidylcholine was first described by Gobley in 1847 as a component of egg yolk, and was initially known as lecithin after the Greek equivalent for egg yolk (lekithos). It was one of the first phospholipids to be elucidated both for structure and synthesis, and has one of the most highly regulated synthetic

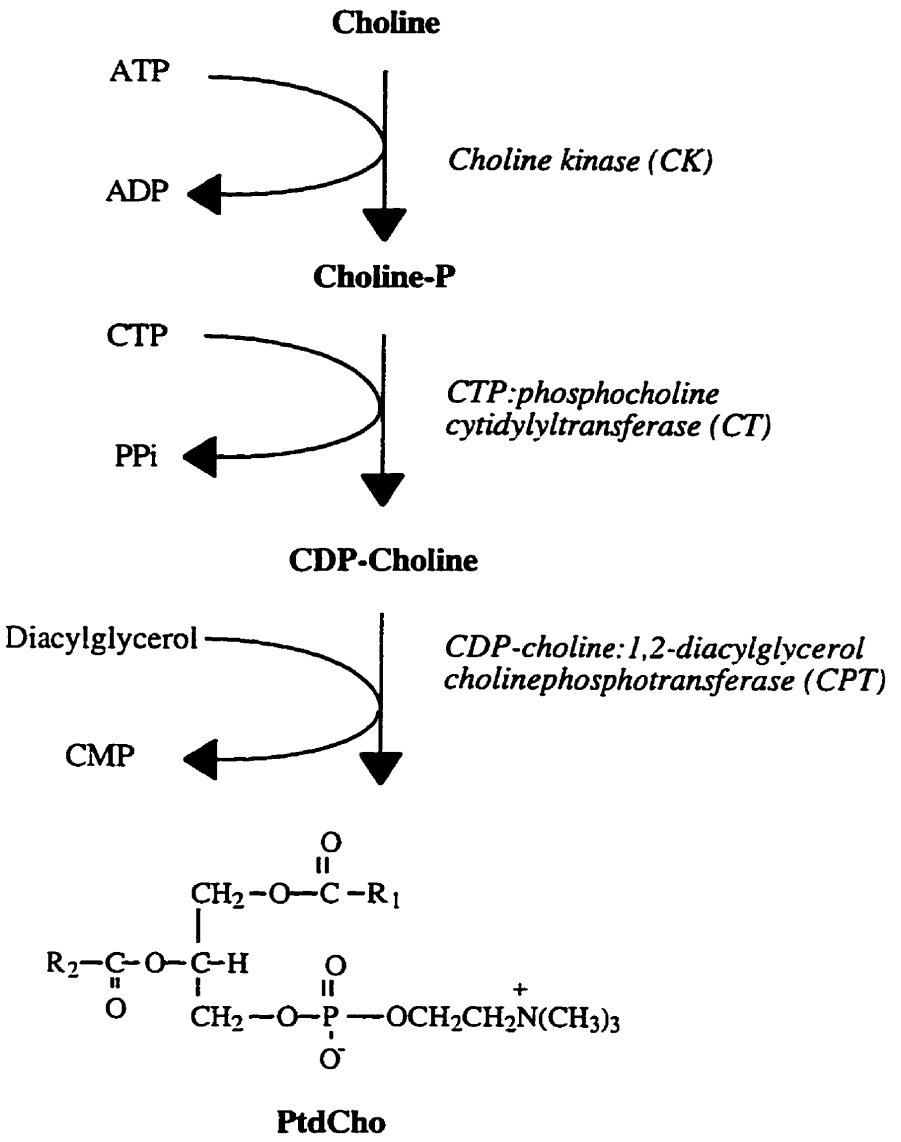

Figure 4. CDP-choline biosynthetic pathway

pathways. PtdCho is the major phospholipid in most cells, comprising up to 50% of the total phospholipid in membranes (White, 1973).

1. Structure

PtdCho is comprised of a glycerol backbone with two fatty acid moieties at the *sn*-1 and *sn*-2 positions, with arachidonic acid or another unsaturated fatty acid found predominately at the *sn*-2 position (Figure 4). The polar head group, phosphocholine is attached at the *sn*-3 position.

2. Biosynthetic pathway

In both hepatic and non-hepatic tissue, PtdCho is synthesized via the Kennedy or CDP-choline Pathway (Figure 4), which involves the addition of a phosphocholine (Pcho) head group to diacylglycerol (Kennedy, 1989). Choline is not synthesized by most mammalian cells and is transported into the cell by both a high and low affinity transporter (Ishidate, 1989; Ishidate and Nakazawa, 1992; Porter and Kent, 1992; Uchida and Yamashita, 1992). Once choline is within the cell, it is rapidly phosphorylated by choline kinase (CK) (Ishidate, 1989). The Pcho is then activated by the addition of a CMP-moiety from CTP. This reaction, which is the rate-limiting step of the pathway, is catalyzed by CTP:phosphocholine cytidylyltransferase (CT) (Weinhold and Feldman, 1992). The final step of the pathway, catalyzed by CDP-choline:1,2-diacylglycerol cholinephosphotransferase (CPT), is the transfer of Pcho from CDP-choline to diacylglycerol resulting in the formation of PtdCho (Cornell, 1989a; Cornell, 1992). In liver, PtdCho can also be synthesized by three sequential *N*-

methylations of PtdEtn, a reaction catalyzed by the PtdEtn methyltransferase (PEMT) (Ridgway and Vance, 1992).

a) Structure of CTP:phosphocholine cytidylyltransferase

CTP:phosphocholine cytidylyltransferase (CT) from rat, hamster and human is a 42 kDa protein containing 367 amino acids that displays a high degree of conservation amongst species (Kalmar et al. 1990; Rutherford et al., 1993; Kalmar et al., 1994; Sweitzer and Kent, 1994). CT is comprised of three domains; the catalytic domain (amino acids 70-250), the lipid binding domain, (amino acids 251-312) consisting of a putative amphipathic alpha helix, and the C-terminal phosphorylation domain (amino acids 313-367) that contains 16 possible phosphorylation sites (Kalmar et al., 1994). The C-terminal has possible phosphorylation sites for cAMP-dependent protein kinase, protein kinase C, cell-cycle protein kinases and casein kinase II (Watkins and Kent, 1990; MacDonald and Kent, 1994; Wang and Kent, 1995b; Wieprecht et al., 1996).

CT does not have N-linked glycosylation sites, a N-terminal leader peptide or lipid modification sequences. It does, however, contain a nuclear localization signal in the N-terminus (amino acids 8-28) (Wang et al., 1995).

b) Regulation of CTP:phosphocholine cytidylyltransferase

CT exists in soluble and membrane-bound forms. In CHO-K1 and HeLa cells the majority of CT was localized to the nucleus, while CT in hepatocytes was primarily cytoplasmic (Terce et al. 1991; Watkins and Kent, 1992; Wang et al., 1993; Wang et al., 1995; Houweling et al., 1996). The CT nuclear localization

signal (amino acids 8-28) was sufficient to direct β-galactosidase into the nucleus, and if mutated, CT was found in both the cytoplasm and nucleus (Wang et al., 1995).

Regulation of CT occurs at the level of enzyme localization, lipid activation, phosphorylation, substrate regulation and enzyme mass (reviewed by Tronchere et al., 1994). A combination of translocation by lipid activators and CT phosphorylation appears to be the main mechanism for enzyme regulation. CT is not lipid modified and does not have transmembrane domains, so membrane association is due to interactions between an amphipathic helix in the lipid binding domain (amino acids 251-312) and the phospholipid bilayer (Cornell and Vance, 1987b; Craig et al., 1994; Dunne et al., 1996). *In vivo* CT present within the cytosol is inactive, and must translocate to the membrane as part of the activation process. This translocation mechanism allows for rapid activation of PtdCho biosynthesis. The *in vivo* signals that initiate translocation have not been fully elucidated, but experimental studies showed that the regulation is most likely a complex balance between phosphorylation state and the presence of specific lipid activators (Yang and Jackowski, 1995). Lipids known to activate CT *in vitro* are the anionic phospholipids, fatty acids and diacylglycerol (Cornell and Vance, 1987a, Arnold and Cornell, 1996). *In vitro*, the presence of PtdCho/oleic acid vesicles stimulated CT activity 3-4 fold and oleate treatment of cultured cells increased PtdCho synthesis (Feldman et al., 1985; Pelech, et al., 1983a). Translocation by lipid activators may occur by the association of CT with a regulatory protein. CT binding protein (CTBP) could act

as such a regulator since it formed complexes with CT *in vitro* upon the addition of oleate to cytosol (Weinhold and Feldman, 1995).

Membrane binding of CT may activate the protein by relieving auto-inhibition by the lipid binding domain, increasing the affinity of the enzyme for CTP or a combination of both (Yang et al., 1995; Wang and Kent, 1995a). *In vitro* and mutagenic studies have shown that the Km for CTP decreased 10-fold upon lipid binding (Yang et al., 1995). This change in enzyme kinetics brought the concentration of CTP required for activity into the physiological range.

The phosphorylation of CT regulates enzyme activity by modifying translocation. The regulatory trend observed was dephosphorylation causes increased CT membrane association and activity (Wieprecht et al., 1994; Yang and Jackowski, 1995; Shiratori et al., 1995; Wieprecht et al., 1996) However, the phosphorylation/dephosphorylation status of CT was not the dominant regulator, as seen from CT mutational analysis (Wang and Kent, 1995b). When all of the CT phosphorylated residues were mutated to alanines, the *in vitro* enzyme activity was not dramatically altered. *In vivo,* these CT mutations displayed increased membrane-associated activity (5-17 fold), but did not have dramatically increased PtdCho synthesis (Wang and Kent, 1995b). Also, 20-60% of the CT in these mutants remained soluble. Mutation of the phosphorylated serines to glutamic acids did not dramatically impair the ability of CT to interact with the membranes (Wang and Kent, 1995b), thus a high degree of negative charges did not preclude CT from associating with membranes. Therefore, the proposed role for phosphorylation is not that it

drives translocation, but that dephosphorylation stabilizes the association of CT with the membrane.

CT activity is also regulated by substrate availability. There appears to be a channeling of the substrates; exogenously added Pcho was not used by CT unless the intracellular concentration of Ca^{2+} was increased (George et al., 1991). The role of channeling in the overall regulation of PtdCho biosynthesis is not understood. The concentration of CTP also regulated activity. As previously mentioned, lipid binding lowered the Km of CT for CTP to physiological ranges. It has also been shown that overexpression of CTP synthase in yeast stimulated the biosynthesis of PtdCho (McDonough et al., 1995).

Feedback regulation by PtdCho has also been demonstrated. In livers or hepatocytes derived from choline-deficient rats, the rate of PtdCho biosynthesis via the CDP-choline pathway was inhibited by 70% compared to choline-supplemented rats. In the choline-deficient hepatocytes, CT translocation to membranes was increased in an attempt to stimulate CT activity, but due to choline deficiency PtdCho synthesis remained decreased. When choline was supplied to choline-deficient hepatocytes, PtdCho synthesis via the CDP-choline pathway increased and membrane-bound CT was released into the cytosol (Vance, 1990; Jamil et al., 1990; Yao et al., 1990). Choline depletion has similar effects on PtdCho metabolism in HepG2 and CHO-K1 (Masatomo et al., 1985; Maeda et al., 1985; Weinhold et al., 1994). Lysophosphatidylcholine (lyso-PtdCho) also inhibits CT activity; lyso-PtdCho binds the lipid binding

domain and competitively inhibits binding of lipid activators, thus deactivating the enzyme (Boggs et al., 1995).

CT protein mass is regulated by gene expression and protein degradation. CT mRNA was shown to be altered by cell cycle; addition of colony stimulating factor to depleted cells initiated the cell cycle and caused a 4-fold induction of mRNA and increased total CT activity (Tessner et al., 1991). Increased CT mRNA was due to increased transcription and mRNA stability. Stimulation of rat liver cells with this growth factor also caused an increase in CT protein mass. Regulation of CT protein degradation was shown in cells treated with cholecystokinin, an agent that stimulates the down-regulation of CT (Matozaki et al., 1991; Groblewski et al., 1995). These studies led to the hypothesis that CT activation by dephosphorylation is followed by degradation. CT translocation appeared to be a one-way event; once translocated to membranes, CT was degraded. This may be an isolated mechanism for down-regulation since treatment of cells with BSA caused translocation of CT from membranes to the cytosol with no apparent degradation (Cornell and Vance, 1987a).

3. Catabolism

The regulation of PtdCho catabolism is likely linked to its synthesis such that the mass of PtdCho within the cell remains constant (Vance, 1990). In models were PtdCho biosynthesis was increased, such as the overexpression of CT in CHO-K1 cells, a reciprocal increase in PtdCho degradation was noted (Walkey et al., 1994). Only in a few cases has it been illustrated that PtdCho degradation was not co-regulated with its synthesis, such as the cholesterol-loaded

macrophage, where initially PtdCho accumulated at the same time CT activity increased. However, CT activity decreased after extensive cholesterol-loading (Shiratori et al., 1994).

PtdCho is catabolized in a pathway that leads to the formation of lyso-PtdCho, due to the activity of phospholipase A_1 and A_2 (Morash et al., 1988; Morash et al., 1989). The lyso-PtdCho is either re-acylated to PtdCho (reviewed by Choy and Arthur, 1990) or degraded further to glycerophosphocholine (GroPcho). The final step of the catabolism pathway is the degradation of GroPcho to choline, which can be recycled back into the synthetic pathway (Morash et al., 1988). This is not the only route of degradation for PtdCho. Phospholipase C and D also degrade PtdCho to form diacylglycerol or phosphatidic acid, respectively. The activities of all three phospholipases are also important in the generation of molecules for signal transduction (Roberts and Dennis, 1990; Tonchere et al., 1994). PtdCho is also selectively degraded to lyso-PtdCho by phospholipase A_2 and then re-acylated with arachidonic acid to alter the fatty acid composition of the PtdCho pool (reviewed Choy and Arthur, 1990).

D. Coordinate regulation of lipid and sterol metabolism

Co-regulation of cholesterol and other lipids is important for cellular physiology. The cholesterol/phospholipid ratio of cellular membranes must remain constant to ensure the correct environment for membrane associated proteins. Altering the amount of cholesterol within a membrane changes lipid packing, bilayer width, permeability and fluidity. Similarly, the content of specific

phospholipids, by virtue of headgroup and fatty acids composition, affects physical characteristics of the membranes such as charge-to-lipid ratio, packing density and polymorphic structure. All of these parameters are important in protein-lipid interactions and regulation of membrane-bound enzymes. Therefore, it is paramount that a mechanism exists by which the cell can regulate the cholesterol/phospholipid ratio, and the phospholipid composition of the cell.

1. Coordinate control of sphingomyelin and cholesterol

The coordinate regulation of SM and cholesterol concentrations has been recognized in both pathological conditions and experimental systems for 20 years or more (reviewed in Kolesnick, 1991). In atherosclerosis, cholesterol, cholesterol ester and SM are all deposited in the artery wall lesions. SM could account for 70-80% of the total phospholipid present in advanced lesions (Smith and Cantab, 1960; Smith, 1974). SM and cholesterol also accumulated as a function of aging and in dietary induced hypercholesterolemia in rabbits (Eisenberg et al., 1969; Barenholz and Thompson, 1980).

The co-regulation of SM and cholesterol is also evident within the Niemann-Pick (NP) disorders (Spence and Callahan, 1989). The NP diseases are lipid storage disorders, of which there are two types. NP Type-I is due to a defect in the lysosomal sphingomyelinase. This defect causes the accumulation of SM, which is paralleled by an increase in cholesterol and other lipids. In NP Type-II the primary defect results in cholesterol accumulation due to defective cellular cholesterol trafficking, but SM also accumulates. Hence, two separate mutations

in the SM and cholesterol metabolic pathways generate similar pathological conditions.

Besides being co-regulated, SM and cholesterol are also co-localized in cells. Both lipids are enriched in the PM compared to the interior organelles (Patton, 1970; Lange et al., 1989) The importance of this co-localization has been shown by studies using bacterial sphingomyelinase to hydrolyze PM SM (Slotte and Bierman, 1988; Gupta and Rudney; 1991). Treatment of various cells with bacterial sphingomyelinase resulted in the movement of cholesterol to the interior of the cell. This cholesterol translocation caused increased ACAT activity, repression of cholesterol synthesis, and increased steroid hormone secretion (Slotte and Bierman, 1988; Gupta and Rudney, 1991; Pörn et al., 1991; Skiba et al., 1996). Recently, it was demonstrated that the suppression of cholesterol synthesis in sphingomyelinase treated cells was directly due to decreased SREBP cleavage and decreased transcription of sterol-regulated genes (Scheeh et al., 1997).

Conversely, artificially increasing the SM content of cultured cells with liposomes stimulated cholesterol synthesis and repressed esterification (Gatt and Bierman, 1980; Kudchodkar et al., 1983). Stimulation of SM synthesis by the addition of ceramide also repressed cholesterol esterification by increasing the level of cholesterol needed to activate ACAT (Okwu et al., 1994).

The co-localization of cholesterol and SM in membranes could be due in part to lipid microdomains found in both the PM and the Golgi. In the PM, caveolae, small invaginations of the membrane that are devoid of clathrin coats,

contain clusters of glycosphingolipids and SM and need cholesterol to function (Parton and Simons, 1995) and may be involved in potocytosis and cell signaling (Anderson, 1993). The Golgi apparatus and PM both contain another type of microdomain known as rafts which are moving platforms of sphingolipids and cholesterol (Simons and Ikonene, 1997). These microdomains are formed within the Golgi membrane and seem to have roles in sorting of resident Golgi membrane proteins from those destined for the PM and in transport of proteins to the PM. The proteins which bind to the lipid rafts include GPI-anchored proteins, transmembrane proteins, doubly acylated tyrosine kinases of the Src family and caveolin. These domains may be maintained due to preferential physical interactions between cholesterol and SM demonstrated in model membranes (Barnenholz, and Thompson, 1980; McIntosh et al., 1992).

One possible mechanism for coordinate control of SM and cholesterol is through dual regulation of biosynthetic or degradative pathways. Studies using CaCo-2 cells showed that those cells enriched in SM had higher rates of synthesis for both cholesterol and long-chain bases. Cells enriched with cholesterol had synthesis rates for cholesterol and long-chain base that were significantly decreased (Chen et al., 1993). Other studies have shown that incubation of cells with LDL modestly inhibited synthesis of SM (Merrill, 1983; Chatterjee et al., 1986).

Previous studies in our lab also showed co-regulation of cholesterol and SM metabolism. First, short-chain ceramide analogues inhibited ACAT activity in CHO cells (Ridgway, 1995a). We have also shown that treatment of CHO cells

with 25-hydroxycholesterol increased SM synthesis due to increased conversion rate of ceramide to SM (Ridgway, 1995b). This regulatory step seemed to occur within the Golgi apparatus. Thus, from these and other studies there seems to be direct metabolic interaction between SM synthesis and cholesterol metabolism which could contribute to covariance of these two lipids.

2. Coordinate control of PtdCho and cholesterol

PtdCho is the major phospholipid in eukaroytic cells, thus it has a key role in maintaining the cholesterol/phospholipid ratio. PtdCho mass remains constant in many experimental conditions (described in section C-3), indicating its importance to cellular functions. However, alterations in cholesterol homeostasis do appear to alter PtdCho synthesis and mass. Treatment of L_6 cells with compactin or 25-hydroxycholesterol, agents that inhibit cholesterol synthesis, resulted in a 50% reduction in PtdCho synthesis (Cornell and Goldfine, 1983). This decrease in PtdCho synthesis is due to a 20-30% inhibition of CT activity. Treatment of $HepG_2$ cells with simvastatin, another cholesterol synthesis inhibitor, also decreased PtdCho synthesis and CT activity (Yanagita et al., 1994). Conversely, cholesterol-loaded macrophages displayed increased PtdCho synthesis, membrane-associated CT activity, and increased enzyme dephosphorylation (Shiratori et al., 1994; Shiratori et al., 1995). Increased PtdCho synthesis was also observed in cells treated with a combination of 25-hydroxycholesterol and the ACAT inhibitor 58035 (Shiratori et al., 1994). Similar results were also seen in the livers of cholesterol-fed rats (Lim et al., 1983). In all of these studies choline kinase and

cholinephosphotransferase activities were not affected. Thus, in these models increased cellular cholesterol caused an increase in PtdCho synthesis, and diminished cholesterol synthesis inhibited PtdCho synthesis.

One contrasting study showed that treatment of LLC-PK cells with 10 μg cholestane-3β,5α,6β-triol/ml (an oxysterol) for 24 h stimulated PtdCho synthesis and CT activity (Mahfouz et al., 1996). This treatment increased cellular uptake of linoleic and oleic acids. Other studies with oxysterols showed effects on fatty acid distribution in phospholipids, and inhibition of archidonate metabolism and prostacyclin synthesis (Seilan and Dubuquoy, 1990; Seilan, 1990). In these instances, the effects of oxysterol on PtdCho synthesis may be due to altered fatty acid concentrations and not decreased cholesterol synthesis.

3. Coordinate control of cholesterol and fatty acid synthesis

The co-regulation of cholesterol and fatty acids has been suggested for twenty years (Limanek et al., 1978), but recently the regulatory mechanism has been elucidated. In 1978, the laboratory of T. Y. Chang developed a CHO cell mutant (MC-1) that was auxotrophic for both cholesterol and unsaturated fatty acids (Limanek et al., 1978). Both defects reverted in parallel suggesting that a single mutation caused both auxotrophies. Metabolic studies also showed that intermediates of the cholesterol biosynthetic pathway, such as mevalonate diphosphate, inhibited both cholesterol and fatty acid synthesis (Ku, 1996).

The coordinate regulation of fatty acids and cholesterol occurs at the level of gene expression. The transcription factor ADD1 (adipocyte determination- and differentiation-dependent factor 1) was cloned due to its ability to regulate lipid

accumulation in the adipocyte. ADD1, is the rat homologue of SREBP-1c and stimulates synthesis of mRNA for fatty acid synthase and acetyl coenzyme A carboxylase (Tontonoz et al., 1993; Bennett et al., 1995; Lopez et al., 1996). Coordinate transcriptional regulation of fatty acid and cholesterol biosynthetic enzymes was also demonstrated in a number of studies that altered cholesterol metabolism. HepG2 cells treated with the squalene synthase inhibitor TAN1607A had decreased free and esterified cholesterol content and increased mRNA levels for fatty acid synthase (Kawabe et al., 1996). The concentration required to activate fatty acid synthase transcription was 10-times higher than that needed to induce synthesis of LDL receptor mRNA. In the same study, fatty acid synthase transcription was increased by ALLN, a calpain inhibitor which inhibits SREBP degradation. Also transgenic mice containing a truncated, constitutively active version of SREBP-1a accumulated triacylglycerol and did not down-regulate cholesterol synthesis (Shimomura et al., 1997). Thus, the regulatory control between fatty acids and cholesterol appears to be due to the ability of SREBP to up-regulate both cholesterol and fatty acid biosynthesis.

4. Regulation of PtdCho biosynthesis by fatty acids

As discussed in Section C-2b, various fatty acids, oleic acid in particular, activates CT activity *in vitro*. Also, when fatty acids are added to cultured cells, PtdCho synthesis is increased due to increased CT translocation to membranes. It is possible that fatty acids or a fatty acid-derived product are the physiological trigger that cause membrane-association.

II. Project Aim

The hypothesis that I set out to test was that the phospholipids, particularly SM and PtdCho, are metabolically regulated by alterations in cholesterol homeostasis. Previous studies had evaluated the covariance of cholesterol and phospholipids either using *in vitro* studies or acute treatment regimes that rapidly alter cholesterol homeostasis. In this study, I have employed CHO cells with well defined mutations in cholesterol biosynthesis and regulation. SRD 6 cells are cholesterol auxotrophs which do not display transcriptional activation of sterol-regulated genes, SRD 2 cells are 25-hydroxycholesterol resistant cells which constitutively transcribe the sterol-regulated genes and SRD 4 cells are ACAT mutants which lack cholesterol esterification and also do not down-regulate transcription of the sterol-regulated genes. These cells were used to determine how alterations in cholesterol regulation affect phospholipid metabolism, and identify the mechanisms involved.

The first part of the study focused on PtdCho biosynthesis and its regulation in the SRD mutants. In cholesterol-loaded macrophages (Shiratori et al., 1994) and livers of cholesterol-fed rats (Lim et al., 1983), it was shown that increased cholesterol content increased PtdCho synthesis. We hoped to determine if decreased or increased cholesterol synthesis also played a role in regulating PtdCho synthesis. In doing so, we hoped to elucidate the mechanism of this regulation.

The second part of the study investigated the regulation of SM synthesis in

SRD cells. In CHO-K1 cells it was shown that 25-hydroxycholesterol stimulated SM synthesis. We focused on this response to 25-hydroxycholesterol and investigated the possible role of the 25-hydroxycholesterol receptor OSBP in this process. We also used pharmacological agents that alter cholesterol homeostasis to determine exactly what aspects of cholesterol regulation affected SM synthesis. The combination of these investigations allowed us to correlate differences in cholesterol content, synthesis, and transport to the effects seen on SM synthesis and OSBP phosphorylation and localization.

III. Materials and Methods

A. Materials

DMEM, FCS, Hind III, Eco RI, Klenow and S1 nuclease (from *Aspergillus oryzae*) were purchased from Gibco/BRL. Pst I was from New England Biolabs. Diacylglycerol kinase (from *E. coli*) was purchased from Cal Biochem. Proline, mevalonate, cholesterol complexed with methyl β-cyclodextrin, methyl β-cyclodextrin, oleic acid, 1,2-dioleoyl-*sn*-glycerol, choline, Pcho, CDP-choline, SM and PtdCho were purchased from Sigma Chemical Co. 25-Hydroxycholesterol was purchased from Steraloids. [^{3}H(G)]-Serine, [1-^{14}C]acetic acid, [9,10-^{3}H]oleic acid, [*methyl*-^{3}H]choline, [*methyl*-^{3}H]choline, [*methyl*-^{14}C]CDP-choline, [*methyl*-^{3}H]Pcho, [α^{32}P]dATP, [γ^{32}P]ATP, [^{32}P]phosphate and Enhance spray were from Mandel Scientific-NEN Life Science Products. Silica Gel 60 and cellulose TLC plates were from E. Merck. Silica G plates were from Fisher Scientific. C_6-[3-^{3}H]ceramide was prepared by T. Lagace as described in Ridgway, 1995b. Kits for measuring cholesterol were purchased from Boehringer-Mannheim. Enhanced chemilluminescence (ECL) kits and μBCA protein determination kits were purchased from Pierce. Lovastatin was from Parke-Davis Pharmaceuticals and U18666A was a generous gift from Dr. M. E. Torkelson of the Upjohn Co. All other chemicals were of reagent grade.

1. Antibodies

The antibody used for OSBP immunochemistry (ab104, prepared by Neale Ridgway) was a polyclonal antibody raised in rabbit to a GST-fusion protein

containing amino acids 201-309 of rabbit OSBP. For immunoblotting and immunofluorescence this antibody was affinity purified using the same GST-fusion protein coupled to Sepharose (Ridgway et al., 1997).

The antibody used for CT immunoblotting and immunoprecipitation (abCT), was a polyclonal antibody raised in rabbit to the full-length CT (Luche et al., 1993; generously provided by Suzanne Jackowski, St. Jude Children's Research Hospital, Memphis, TN).

FITC-goat anti-rabbit secondary antibody was from Organon Teknika/Cappell. FITC-lentil lectin (from *Lens culinaris)* was obtained from Sigma. The goat-antirabbit-HRP secondary antibody was purchased from Gibco/BRL.

2. Preparation of lipoprotein deficient serum (LPDS)

FCS was adjusted to a density of 1.21 g/ml with solid NaBr (290 g NaBr/L). Thirty ml of serum was overlaid with 1.21 g NaBr/ml density solution in a quick seal centrifuge tube. The serum was centrifuged for 26 h at 200,000 x g in a Ti50.2 rotor at 4°C. The tubes were sliced at the boundary of the plasma and overlay solution and the lower portion containing the lipoprotein-free serum was pooled. The LPDS was dialyzed against 10 mM sodium phosphate (pH 7.4) with 0.9% NaCl (w/v) for 48 h (Goldstein et al., 1983). LPDS was filtered through a 0.2 micron polyethersulfone filter (Nalgene) and stored at -20°C.

3. Cell culture

The characteristics of the cell lines used in this study, CHO-K1 (ATCC CCL61), CHO 7, SRD 2, SRD 4 and SRD 6, are briefly described in Table 1.

Table 1. CHO and SRD cell lines

Cell Line	Phenotype	Mutation	Ref.
CHO-K1	wild-type	—	ATCC CCL61
CHO 7	parental line for SRD cells, adapted for growth in LPDS.	—	Metherall et al., 1989 Dawson et al., 1991 Evans and Metherall, 1993
SRD 2	25-OH resistant transcription of sterol regulated genes.	Genomic rearragement of SREBP-2. Fusion of the transcription factor domain to an unidentified protein. -Constitutively active.	Metherall et al., 1989 Dawson et al., 1991
SRD 4	25-OH resistant transcription of sterol regulated genes	Point mutation in SCAP.-Constitutive cleavage of SREBPs	Metherall et al., 1991 Cao et al., 1996 Nohturfft et al., 1996
	ACAT deficient	Point mutation in ACAT. -No activity.	
SRD 6	cholesterol auxotroph	–Defective proteolytic cleavage of SREBP at second site.	Evans and Metherall, 1993 Jackson et al., 1996 Sakai et al., 1996

Cells were grown in monolayers at 37°C in an atmosphere of 5% CO_2. CHO-K1 cells were maintained in DMEM containing 5% (v/v) FCS and 33 μg proline/ml (medium A). CHO 7 cells were maintained in DMEM containing 5% LPDS and 33 μg proline/ml (medium B). SRD 2 and SRD 4 cells were maintained in medium B containing 0.5 μg 25-hydroxycholesterol/ml (medium C). SRD 6 cells were maintained in medium B containing 1 mM mevalonate (0.77 M stock prepared in 10 mM potassium phosphate buffer pH 4.5) and 2 μg cholesterol/ml complexed with methyl β-cyclodextrin (medium D). Stock cultures of CHO 7 and SRD cells were also supplemented with 0.25% (v/v) FCS. Cells were subcultured in 60-mm and 100-mm dishes in 3 ml and 8 ml medium, respectively, but without 0.25% (v/v) FCS. On day 2 or 3, cells received new medium and experiments were started 18-24 h later (refer to figure legends for specific details). 25-Hydroxycholesterol was prepared as a 2.5 mg/ml stock solution in ethanol and added to warm medium (ethanol concentrations in medium did not exceed 0.2%, v/v).

4. Pharmacological agents used to alter cholesterol homeostasis

In this study we used two hypocholesterolemic drugs to alter cholesterol synthesis and trafficking in CHO-K1 cells. Lovastatin, a HMG-CoA reductase inhibitor (Grundy, 1988), was converted from the lactone to a sodium salt by heating at 50 °C in 0.1 N NaOH. The solution (5 mM) was adjusted to pH 7.7 with HCl and aliquots were stored at -20°C (Brown et al., 1978). U18666A, a hydrophobic amine which blocks intracellular cholesterol transport and

cholesterol synthesis by inhibiting squalene cyclase (Phillips and Avigan, 1963; Liscum and Faust, 1989), was added to warm medium from stock solutions dissolved in DMSO (DMSO concentrations in medium did not exceed 0.2%, v/v).

B. Methods

1. Metabolic labeling and mass analysis of phospholipids, sphingolipids, cholesterol and their metabolites.

a) Extraction and analysis of [^{3}H]serine-labeled sphingolipids

Following [^{3}H]serine labeling (refer to Figure legends for times and specific activity), cells were rinsed once with ice-cold phosphate-buffered saline (PBS; 150 mM NaCl, 10 mM sodium phosphate, pH 7.4), and were scraped into 1 ml methanol/water (5/4, v/v). Extracts from two dishes were combined, sonicated on ice for 30 s and aliquots were taken for protein determination (Lowry et al., 1951). Five ml chloroform/methanol (1/1, v/v) and 3 ml 0.58% (w/v) NaCl were added to each tube, shaken vigorously and phases were separated by centrifugation at 2,000 x g for 5 min. The organic phase was extracted twice with 2 ml ideal upper phase (IUP; methanol/0.58% NaCl/chloroform, 45/47/3, v/v) and dried over a 1 ml column of anhydrous sodium sulfate.

Total lipids were resolved from an aliquot of the organic phase by TLC on silica gel 60 plates in chloroform/methanol/acetic acid/water (60/40/4/1, v/v). PtdSer, PtdEtn and SM were visualized by exposure to iodine vapor. Lipids were scraped from the thin layer chromatography (TLC) plates and radioactivity

was quanitated by liquid scintillation counting (Ridgway, 1995b).

To isolate [^{3}H]sphingolipids, aliquots of total lipid extract were dried under N_2, and hydrolyzed in 0.5 ml 0.1 M KOH in methanol at 37°C for 1 h to degrade glycerophospholipids. The remaining lipids were extracted from the hydrolysate with 3 ml chloroform/methanol (2/1, v/v) and 2 ml 0.58% NaCl and neutralized with 100 µL 0.5 N HCl. After the organic phase was extracted twice with 2 ml IUP, lipids were resolved by TLC on silica gel 60 plates in chloroform/methanol/water (65/25/4, v/v). Radiolabeled SM, GlcCer, and Cer were visualized by fluorography. The TLC plates were sprayed with Enhance and exposed to Kodak XAR film for 3 to 4 days at -70°C. Using the fluorograms as a template, lipids were scraped from the TLC plates and radioactivity was quanitated by liquid scintillation counting (Ridgway, 1995b).

b) Extraction and analysis of [^{3}H]choline-labeled phospholipids and metabolites

Following [^{3}H]choline labeling (refer to Figure legends for times and specific activity), cells were rinsed and harvested as described in Section 1a but dishes were scraped into 2 ml of methanol/water (5/4, v/v) instead of 1 ml. Four ml of chloroform was added, shaken vigorously, and phases were separated by centrifugation at 2,000 x g. The organic phase was extracted twice with 2 ml IUP and dried over anhydrous sodium sulfate as previously described. The aqueous phase was saved for [^{3}H]choline metabolite analysis. [^{3}H]Choline labeled PtdCho and SM were separated by TLC on silica gel 60 plates in a solvent system of chloroform/methanol/water (65/25/4, v/v) and visualized by exposure

to iodine vapor. PtdCho and SM were scraped from the TLC plates and radioactivity was quanitated by scintillation counting.

Aqueous [^{3}H]choline metabolites were separated by TLC on silica G plates in ethanol/water/ammonia (48/95/6, v/v). Choline, Pcho, GroPcho and CDP-choline were visualized by spraying plates with 1% phosphomolybdic acid in chloroform/ethanol (1/1, v/v) and 1 % stannous chloride in 3 N HCl, and scraped into vials containing 0.5 ml water for liquid scintillation counting (Morash et al., 1988).

c) Analysis of [^{3}H]thymidine incorporation into DNA

Following [^{3}H]thymidine labeling, cells were rinsed once with ice-cold PBS, twice with 2 ml ice cold 5% (w/v) trichloroacetic acid (TCA), once with 2 ml cold distilled water, and solubilized by incubating at room temperature in 1 ml 0.25 N NaOH for 1 h. The extracts were neutralized with 50 µl of 5 N HCl and radioactivity was quantified by liquid scintillation counting.

d) Analysis of [^{35}S]methionine incorporation into protein

Following [^{35}S]methionine labeling, cells were rinsed twice with ice-cold PBS, solubilized in 1 ml 0.5 N NaOH by incubation at room temperature for 1 h, and aliquots of the cellular extract were spotted on 3 mm Whatmann filters. After drying, the filters were washed twice for 20 min in 10% (w/v) cold-TCA, twice for 20 min 5% (w/v) cold-TCA, once for 5 min in cold 70% (v/v) ethanol and once for 5 min in cold 100% ethanol. The filters were dried and radioactivity was quanitated by liquid scintillation counting.

e) Phospholipid and phosphocholine mass analysis

Separation and detection of phospholipids and cellular Pcho for mass analysis was by TLC as described in Sections 1a and 1b. Samples were scraped into glass tubes and phosphorus was quantitated by digesting the samples with 0.5 ml 70% (v/v) perchloric acid for 45 min at 120° C. After samples had cooled, 3.5 ml water, 0.5 ml 2.5% (w/v) ammonium molybdate and 0.5 ml of 10% (w/v) ascorbic acid were added, test tubes were heated for 5 min at 80-90°C and absorbance was read at 820 nm. The amount of phosphorus present was determined by linear regression from a standard curve (Rouser et al., 1966).

f) Diacylglycerol mass analysis

Lipid extracts were prepared as described in Section 1a, an aliquot (20% of total extract) was dried under N_2 and 20 µL of a 7.5% (w/v) octylglucoside-5 mM cardiolipin solution (solubilized by sonication in 1 mM DETAPAC) was added. The samples were place in a bath sonicator for 15 s and incubated at room temperature for 10 min. The assay was performed in 100 µl of 50 mM imidazole-HCl (pH 6.6), 50 mM NaCl, 12.5 mM $MgCl_2$, 1 mM EDTA, 2 mM DTT, 5 µg diacylglycerol kinase (10 µl, 6.3 U/mg, 0.5 mg/ml) in 5 mM imidazole, pH 6.6) and 10 nmol of [γ^{32}P]ATP in 100 mM imidazole and 1 mM DETAPAC, (1 x 10^5 dpm/nmol). The reaction was incubated for 45 min at room temperature and terminated by the addition of 3 ml chloroform/methanol (1:2, v/v), 0.7 ml of 1% (v/v) $HClO_4$, 1 ml chloroform and 1 ml 1% (v/v) $HClO_4$. The phases were separated by centrifugation at 2,000 x g and the organic phase was extracted

twice with distilled water and dried under N_2. An aliquot of the lipid extract was separated by TLC on pre-dried (60 min at 110°C) silica gel 60 plates in chloroform/methanol/acetic acid (65/15/5 v/v). Phosphorylated lipids were visualized by exposure to Kodak XAR film for 2-4 h at -70°C and scraped from the TLC plates using the autoradiogram as a template. Radioactivity was quantitated by liquid scintillation counting. Diacylglycerol mass was determined by linear regression from a standard curve (Preiss et al., 1986).

g) Extraction and analysis of [^{14}C]acetate-labeled sterols and fatty acids

Following [^{14}C]acetate labeling (refer to Figure legends for times and specific activity), medium was transferred to screw-capped tubes and cells were dissolved in 1 ml 0.1 N NaOH. After an aliquot of cell lysate was removed for protein determination (Lowry et al., 1951), cellular extracts were combined with medium, 3 ml ethanol and 0.5 ml 50% (w/v) KOH were added and lipids were hydrolyzed by incubation at 60 °C for 1 h. Sterols were extracted with hexane (4 ml) and resolved by TLC on silica gel 60 plates in petroleum ether/diethyl ether/acetic acid (60/40/1, v/v) (Brown et al., 1978). Radioactivity was quantitated using a Bioscan 200 radio-imaging scanner. The fatty acids were isolated from the aqueous phase by acidification with HCl (to pH<3) and hexane (4 ml) extraction. Fatty acids were resolved using the same TLC system as above, and visualized using iodine vapor. Radiolabeled-fatty acids were quantitated by scraping and liquid scintillation counting.

2. Enzyme Assays

a) Cellular fractionation for analysis of PtdCho biosynthetic enzymes

Cells were harvested in ice-cold PBS and sedimented at 2,000 x g for 5 min. Cell pellets were homogenized in 20 mM Tris-HCl (pH 7.4), 0.1 mM PMSF, 10 mM NaF, 1 mM EDTA, and 5 mM DTT (Buffer A, 50 µl/100 mm dish) by 40 strokes in a glass dounce and two 5 s bursts with a needle probe sonicator (40% power setting). Homogenates were centrifuged for 1 h at 130,000 x g, the supernatant (cytosol) was collected and the pellet (total membranes) was re-suspended in buffer A containing 0.25 M sucrose (50µl /100 mm dish). Protein concentrations for these fractions were determined using the Lowry method (Lowry et al., 1951).

b) Choline kinase

Cytosolic fractions (20-60 µg protein) were assayed for choline kinase activity in 100 µl of 100 mM Tris-HCl (pH 8.5), 10 mM $MgCl_2$, 10 mM ATP and 1 mM [*methyl*-^{3}H]choline (2 µCi/mmol). The reaction was incubated for 20 min at 37°C and terminated by boiling the test tubes for 2 min (Pelech et al., 1983b). [^{3}H]Pcho was separated from [*methyl*-^{3}H]choline by TLC on silica G plates in ethanol/water/ammonia (48/95/6, v/v). Pcho and choline were visualized and quantitated using the procedure described in Section 1b.

c) Cholinephosphotransferase

Membrane fractions (40-80 µg protein) were assayed for cholinephosphotransferase in 50 µl of 50 mM Tris-HCl (pH 8.5), 10 mM $MgCl_2$,

0.5 mM EGTA, 0.4 mM [*methyl*-^{14}C]CDP-choline (0.5 Ci/mol) and 2.4 mM 1,2-*sn*-diolein (prepared as a diolein-PtdCho 1:5 (w/w) emulsion in 0.05% (w/v) Tween-20). The reaction was incubated for 15 min at 37 °C and terminated by the addition of 1.5 ml chloroform-methanol 1:2 (v/v), 0.8 ml water, and 0.5 ml chloroform. [^{14}C]PtdCho in the organic phase was extracted twice with IUP, dried under N_2, and quantitated by liquid scintillation counting of an aliquot (Cornell, 1992).

d) CTP:phosphocholine cytidylyltransferase

Cytidylyltransferase activity was assayed in both cytosolic and membrane fractions (20-70 µg protein) in 50 µl of 50 mM Tris-HCl (pH 7.4), 0.1 mM NaCl, 10 mM $MgCl_2$, 3 mM CTP, and 1.5 mM [*methyl*-^{3}H]Pcho (1 Ci/mol), in either the presence or absence of 0.1 mM:0.1 mM PtdCho-oleic acid vesicles (aqueous suspension prepared by sonication). The reaction was incubated at 37°C for 25 min and terminated by the addition of 1 volume methanol/ammonia (9/1, v/v) (Cornell, 1989b). [^{3}H]CDP-choline was isolated and quantitated as described in Section 1b.

e) Cellular fractionation for analysis of SM biosynthetic enzymes

Cells were rinsed once in ice-cold PBS, scraped in ice-cold PBS, and collected by centrifugation at 2,000 x g for 5 min. Cells were homogenized in 20 mM Tris-HCl (pH 7.7) and 10 mM EDTA (buffer B) by 40 strokes of a dounce homogenizer. The membrane fraction was isolated by centrifugation at 100,000 x g for 1 h and was re-suspended in buffer B (20 µl/100 mm dish).

f) SPT

SPT activity was assayed in the membrane fraction (50-120 μg protein) in a final volume of 100 μl of 100 mM Hepes (pH 8.0), 2.5 mM EDTA, 5 mM DTT, 50 μM pyridoxal phosphate, 200 μM palmitoyl-CoA and 2 mM [^{3}H]serine (100 dpm/pmol) for 10 min at 37°C. [^{3}H]Ketosphinganine was extracted with 0.2 ml 0.5 N NH_4OH, 1.5 ml chloroform/methanol (1/2, v/v), 1 ml chloroform and 2 ml 0.5 N NH_4OH (Merrill, 1983). The organic phase was extracted twice with IUP and dried under N_2. The product was isolated by TLC on silica gel 60 plates in chloroform/methanol/2 N NH_4OH (40/10/0.5, v/v) and visualized using fluorography as described in Section 1a. [^{3}H]Ketosphinganine was quantitated by scraping using the fluorogram as a template and liquid scintillation counting.

g) SM synthase

SM synthase activity was measured in membranes (50-120 μg) in a final volume of 500 μl of 25 mM KCl, 500 μM EDTA, 50 mM Tris-HCl (pH 7.4), and 20 μM [^{3}H]C_6Cer:BSA (1:1 mol/mol; 100 dpm/pmol) for 10 min at 37 °C. Assays were terminated with 4 ml chloroform/methanol (1/2, v/v), extracted with 2 ml 0.5% NaCl and dried under N_2 (Futerman and Pagano, 1992). [^{3}H]SM was separated by TLC on silica gel 60 plates in chloroform/methanol/15 mM $CaCl_2$ (60/35/8, v/v) and visualized by fluorography as described in Section 1a. Radioactivity was quantitated by scraping and liquid scintillation counting.

h) ACAT

ACAT activity in intact cells was measured by [^{3}H]oleate incorporation in cholesteryl ester (Goldstein et al., 1983). The [^{3}H]oleate/BSA substrate (10 mM

oleate) was prepared by re-suspension of 500 μCi [^{3}H]oleic acid (dried under N_2) in 10 ml of 9.3 mM sodium oleate, 12% (w/v) BSA (fatty acid free), and 150 mM NaCl. Cells were labeled with 100 nmol [^{3}H]oleate/BSA/ml medium for the last hour of experimental protocols. After labeling with [^{3}H]oleate/BSA, cells were washed twice with ice-cold 150 mM NaCl, 50 mM Tris-HCl (pH 7.4), 2 mg BSA/ml and once with 150 mM NaCl, 50 mM Tris-HCl (pH 7.4). Lipids were extracted from the dishes by incubation in 2 ml hexane/isopropanol (3/2, v/v) for 30 min at room temp. Solvent was removed and cells were rinsed with an additional 1 ml of hexane/isopropanol. Extracts were combined, dried under N_2 and dissolved in 50 μl hexane. Lipids were resolved by TLC on silica gel 60 plates in hexane/diethyl ether/acetic acid (90/30/1, v/v). Radiolabeled triglycerides and cholesteryl oleate were visualized with iodine vapor, scraped and radioactivity quantitated by liquid scintillation counting. After the removal of solvent from the dishes, cell protein was solubilized in 0.1 N NaOH and aliquots were assayed for protein (Lowry et al., 1951).

3. Measurement of mRNA levels

a) Preparation of CT probe for S1 nuclease protection assays

The plasmid pCMV5CT containing the rat liver CT cDNA (Kalmar et al., 1990) was digested with Hind III and Eco RI to yield a 96 bp fragment containing the first 86 bp of the CT coding region. This fragment was ligated into Hind III/Eco RI digested M13 mp19 and used to transform DH5αF' cells by electroporation. Positive transformants were identified by blue/white selection

Table 2: Restriction enzymes and hybridization conditions used for S1 nuclease protection assays

Protein	Restriction Enzyme	Hybridization Conditions
HMG-CoA reductase	Eco RI	heat to 90°C, slow cool to 52°C.
Farnesyl diphosphate synthase	Eco RI	heat at 80°C for 10 min, place at 37°C.
LDL receptor	Pst I	heat to 90°C, slow cool to 52°C.
HMG-CoA synthase	Hind III	heat at 80°C for 10 min, place at 37°C.
CTP:phosphocholine cytidylyltransferase	Hind III	heat to 90°C, slow cool to 37°C.
Glyceraldehyde-3-phosphate dehydrogenase	Eco R1	heat at 80°C for 10 min, place at 37°C.

and several M13 clones were sequenced to identify those with the correct inserts. Single-stranded M13 phage for probe preparation was prepared by inoculating a 50 ml culture of TG-1 cells in LB with a positive M13 colony. Single stranded M13 was isolated from the culture supernatant by PEG precipitation (Messing, 1983), and phenol extraction. Preparation of S1 probes for measuring sterol-regulated mRNAs were prepared as previously described (Ridgway and Lagace, 1995)

b) RNA extraction

Cells were homogenized in 4 M guanidine isothiocynate, 25 mM sodium citrate (pH 7.0), 0.5% (w/v) sarcosine and 0.1 M β-mercaptoethanol (solution A) by 3 passages through an 18 gauge needle. RNA was extracted by the addition of a 1/10 volume of 160 mM sodium acetate (pH 4.0), 1 volume of water-saturated phenol and 0.2 volumes of chloroform/isoamyl alcohol (49/1, v/v). After vigorous shaking and incubation on ice for 15 min, samples were centrifuged for 15 min at 9,800 x g to separate the phenol and aqueous phases. RNA was precipitated from the aqueous phase by incubation with 1 volume of isopropanol at -20 °C for at least 2 h. The samples were centrifuged for 15 min at 9,800 x g, and the RNA pellet was re-dissolved in a 1/5 volume of solution A. The RNA was extracted twice with phenol/chloroform (1/1, v/v), once with chloroform and ethanol precipitated. RNA samples were stored in ethanol at -20 °C (Chomczynski and Sacchi, 1987).

c) Preparation of S1 nuclease probes

Single stranded M13 clones were used as templates to prepare ^{32}P-labeled

single stranded DNA probes in the following manner. The universal primer (5'-d[GTAAAACGACGGCCAGT]-3', 8 ng) was annealed to 1.5 µg single stranded M13 template (55 °C for 15 min, followed by cooling at room temp for 10 min). The extension reaction was for 30 min at 37°C, in 50 mM NaCl, 10 mM Tris-HCl (pH 7.5), 1.3 mM $MgCl_2$, 250 µM dCTP, 250 µM dGTP, 250 µM dTTP, 3 µM dATP, 100 µM [α-^{32}P]dATP (800 µCi/mmol) and 3-5 U of Klenow. An additional 2.5 nmol dNTPs and 3-5 U Klenow were added and incubated for 10 min at 37°C. The reaction was stopped by heating at 65°C for 10 min. The extension reaction was digested with a unique restriction enzyme (refer to Table 2) that cut at the 3'end of the cDNA insert to release the single-stranded probe. Single-stranded probes were isolated by electrophoresis in 5% acrylamide/7 M urea gels run at 300 V in 0.5 X Tris/Borate/EDTA for approximately 2 h, and identified by autoradiography with Kodak XAR film for 1 min. Probes were cut from the gel using the autoradiogram as a template, and were eluted from the crushed gel by overnight incubation at 37°C in 0.5 M ammonium acetate (pH 7.0), 10 mM magnesium acetate, 0.1% (w/v) SDS and 1 mM EDTA. The probes were extracted twice by phenol/chloroform (1/1 v/v), once with chloroform and ethanol precipitated with 30 µg yeast tRNA as a carrier. Purified probes were dissolved in TE (10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0) (Chin et al., 1985).

d) S1 nuclease protection assay

RNA and the S1 nuclease probes were combined (see figure legends for specific amounts), dried in a speed vac, and re-suspended in 30 µl of 40 mM PIPES (pH 6.4), 1 mM EDTA, 0.4 M NaCl and 80% (v/v) formamide.

Hybridization was carried out for 16-18 h under the conditions described in Table 2. Excess probe was digested with 500 U of S1 nuclease in 50 mM sodium acetate (pH 4.5), 400 mM NaCl, 4.5 mM $ZnSO_4$ and 20 µg/ml salmon sperm DNA in a final volume of 250 µl at 37°C for 45 min, and stopped by the addition of 100 µl of 3 M ammonium acetate (pH 7.0), 100 mM EDTA and 200 µg/ml yeast tRNA. The samples were ethanol precipitated and dried. Pellets were re-dissolved in S1 loading dye (90% (v/v) formamide, 0.2% (w/v) bromophenol blue, 0.2% (w/v) xylene cyanol, and 50 mM NaOH), denatured at 90 °C for 5 min and run on a 5% acrylamide/ 7M urea gel at 300V (Metherall et al., 1989). The gel was fixed in 10% (v/v) methanol, 10% (v/v) acetic acid, dried, and exposed to Hyperfilm (Amersham) at -70°C for 3-18 h. mRNA was quantitated from autoradiograms by scanning and analysis on a Macintosh Apple ONE Scanner using the NIH Image software package (version 1.55).

4. Immunochemistry

a) Preparation of samples for CT immunoblotting and immunoprecipitation

Cytosol and membrane fractions were isolated as described for enzyme assays in Section 2a. Membranes were re suspended in 150 µl buffer A containing 1% (v/v) Nonidet NP-40 and incubated on ice for 15 min. The soluble fraction was collected by centrifugation for 15 min at 15,000 x g. Total cell extracts were prepared by solubilization of cell pellets in buffer A containing 1 % Nonidet NP-40 and the soluble fraction was collected after centrifugation as described above.

For immunoprecipitation, cells were fractionated into cytosol and membranes, as described in Section 2a, in buffer A containing 100 nm okadaic acid and 100 μM sodium vanadate. To both fractions, a final concentration of 0.3% (w/v) Triton X-100 was added and samples were incubated on ice 15 min prior to centrifugation for 15 min at 15,000 x g.

b) Preparation of samples for OSBP immunochemistry

Cells were harvested in ice-cold PBS and collected by centrifugation in a microfuge (15,000 x g for 15 s). Pellets were solublized 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, 2 μg aprotinin/ml, 2.5 μg leupeptin/ml (buffer C) and 0.3% (w/v) Triton X-100 on ice for 30 min followed by centrifugation at 4°C for 15 min at 15,000 x g. The supernatant fraction was collected and used for immunoblotting and immunoprecipitation (Ridgway et al., 1997).

c) Immunoblotting and immunoprecipitation of OSBP and CT

For immunoblotting, equal amounts of protein (10-30 μg) were separated on either a 10 % (CT) or 6% (OSBP) acrylamide gel by SDS-PAGE and transferred in 25 mM Tris-HCl, 192 mM glycine and 20% (v/v) methanol to nitrocellulose membrane by electrophoresis at 100 V for 1 h (Towbin et al., 1979). The nitrocellulose filter was blocked for 12-48 h in Tris-buffered saline (TBS; 20 mM Tris-HCl pH 7.4, 150 mM NaCl) with 0.1% (v/v) Tween 20 and 5% (w/v) skim milk powder (Blotto). The filter was then incubated for 2 h in Blotto containing a

1/1,000 dilution of the primary antibody (ab104 or abCT). After 3-4 washes with Blotto, the filter was incubated with a goat-antirabbit antibody coupled to horse radish peroxidase (1/5,000 dilution) for 1 h (Ridgway et al., 1997, for OSBP). The filter was washed 3-4 times with Blotto and once with TBS and developed by the enhanced chemilluminescence method according to the manufacturers instruction.

Immunoprecipitation using equal amounts of protein (30-40 µg) was done in 200 µl of buffer C with 1% (w/v) Triton X-100 containing 1/100 (Ab104) or 1/200 (AbCT) dilution of primary antibody for 1-2 h at 4° C. The immune complexes were isolated by incubation with 40 µl of a 1:1 (v/v) slurry of protein A-Sepharose in PBS containing 1% (w/v) Triton X-100 for 30-45 min at room temperature. Protein A-Sepharose beads were washed 6-8 times with 0.5 ml PBS containing 1% (w/v) Triton X-100. Immunoprecipitated proteins were separated by SDS-PAGE on 6% or 10% acrylamide gels. [^{32}P]labeled OSBP or CT was visualized by autoradiography with Amersham Hyperfilm at -70 °C.

d) Indirect immunofluorescence of OSBP

Cells grown on glass coverslips were fixed in 3 % (v/v) formaldehyde in 10 mM sodium phosphate (pH 7.4), 225 mM NaCl, 2 mM $MgCl_2$ (buffer D) for 15 min at room temperature. The coverslips were washed twice with buffer D containing 5 mM ammonium chloride. Cells were then permeabilized by incubation in buffer D containing 0.05% (w/v) Triton X-100 for 10 min at -20 °C. Cells were blocked by incubation with buffer D containing 1% BSA (buffer E) for 15 min at room temp. Cells were treated with a 1/1,000 dilution of primary

antibody (ab104) for 1 h at 37 °C in buffer E. Cells were washed 3 times with buffer E for 15 min at room temperature, followed by incubation with the secondary antibody, FITC-labeled goat anti-rabbit antibody (3.5 μg/ml in buffer E) for 45 min at 37 °C. After 3 washes with buffer E and 1 wash with distilled water, coverslips were mounted on slides with 2.5% (w/v) 1,4-diazadicyclo-[2.2.2]-octane in 50 mM Tris-HCl (pH 9.0) and 90 % (v/v) glycerol (Ridgway et al., 1992). Fluorescence microscopy was performed on a Olympus microscope using a 100x oil immersion objective and photographed with Kodak TMY-400 black and white film.

e) Indirect Immunofluorescence with FITC-lentil lectin

Cells grown on coverslips were fixed, permeabilized and blocked as described in Section 4d. Cells were incubated for 45 min at 37°C with 1 ml buffer E containing 2 μg FITC-lentil lectin, followed by three 15 min washes with buffer E and one wash with distilled water. Coverslips were mounted and photographed as described in Section 4d (Ridgway et al, 1989).

f) Phosphopeptide mapping of OSBP

[^{32}P]Phosphorus labeled OSBP was immunoprecipitated as described in Section 4c and transferred to PVDF by electrophoresis at 100 V for 2 h in 25 mM Tris, 192 mM glycine and 10% (v/v) methanol. The radioactive bands were visualized by autoradiography, cut from the filter, placed in a 1.5 ml eppendorf tube and washed once with methanol and 5 times with distilled water. The membrane was blocked by incubation with 0.5% (w/v) polyvinylpyrolidone-360 in 100 mM acetic acid at 37 °C for 30 min. The filter segments were washed 10

times with distilled water and 5 times with 50 mM ammonium bicarbonate and 5% (v/v) acetonitrile (buffer F). Protein affixed to the filter segments was digested in 200 µl buffer F containing 40 µg TPCK-trypsin/ml for 3-6 h at 37 °C, followed by incubation for 12-18 h with an additional 20 µg of trypsin. The supernatant was transferred to a new tube, dried on a speed vac, re-suspended once in 100 µl distilled water and dried again. The resultant pellets were dissolved in 10 µl electrophoresis buffer (10% (v/v) acetic acid and 1% (v/v) pyridine) and spotted on 0.25 mm cellulose TLC plates. Two dimensional separation was performed by electrophoresis for 45 min at 750 V, followed by TLC in pyridine/water/*n*-butanol/acetic acid (30/24/20/6, v/v) (Shiratori et al., 1995). [^{32}P]Phosphopeptides were visualized by autoradiography using Kodak XAR film at -70°C for 3-8 days.

IV. Results

A. Characterization of SRD Lines

1. Alterations in sterol-regulated gene expression

SRD cells are unique models in which to study the effects of altered cholesterol homeostasis on phospholipid and sphingolipid metabolism. The following experiments were performed to confirm the phenotypes of the three SRD cell lines and the control cells. As shown in Figure 5 and Table 1, all three SRD cell lines have mutations that effect SREBP processing, thus altering expression of sterol-regulated genes. These variations in mRNA levels for the sterol-regulated genes were shown by S1 nuclease protection assays (Figure 6). As shown previously (Metherall et al., 1989; Dawson et al., 1991; Evans and Metherall, 1993), the parental cell line (CHO 7) had normal transcriptional suppression of the HMG-CoA synthase, farnesyl diphosphate synthase, LDL receptor and HMG-CoA reductase genes by 25-hydroxycholesterol. GAPDH mRNA did not vary with 25-hydroxycholesterol or cholesterol treatment and therefore was used as an internal load control for the standardization of sterol-regulated gene expression.

The cholesterol auxotrophic SRD 6 cells have a defect in the second, non-sterol regulated proteolytic cleavage of SREBP-1 and 2, which renders the SREBPs constitutively membrane bound (Sakai et al., 1996). The SREBP transcription factors are unable to migrate to the nucleus, and therefore cannot activate transcription of the sterol-regulated genes. As previously shown (Evans

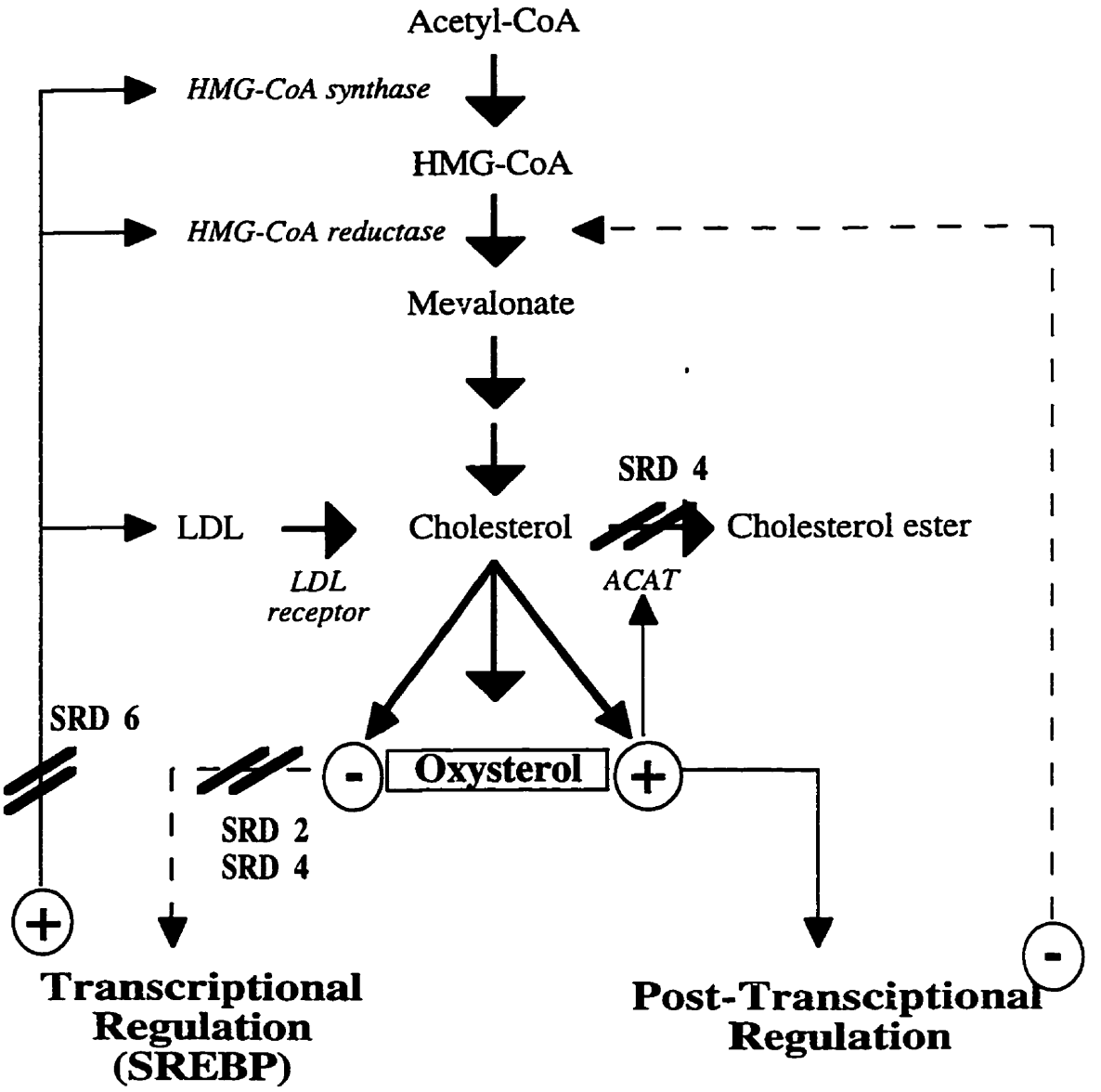

Figure 5. Altered cholesterol regulation in SRD cells.

Figure 6. Sterol-regulated gene expression in CHO 7 and SRD cells. mRNA was isolated from cells grown for 24 h in medium B (without cholesterol), prior to treatment with either 25-hydroxycholesterol (2.5 μg/ml, +) dissolved in ethanol or solvent alone (-). S1 nuclease protection assays were done as described in Materials and Methods. Thirty micrograms (HMG-CoA reductase and LDL receptor), 15 μg (FPP-synthase) or 10 μg (HMG-CoA synthase and GAPDH) of total mRNA was assayed with 75,000 cpm (reductase and FPP synthase), 60,000 cpm (LDL receptor), 50,000 cpm (synthase) or 30,000 cpm (GAPDH) of single stranded [^{32}P]dATP labeled DNA probe. The autoradiograms are the result of 3-24 h exposures at -70°C using Amersham Hyperfilm.

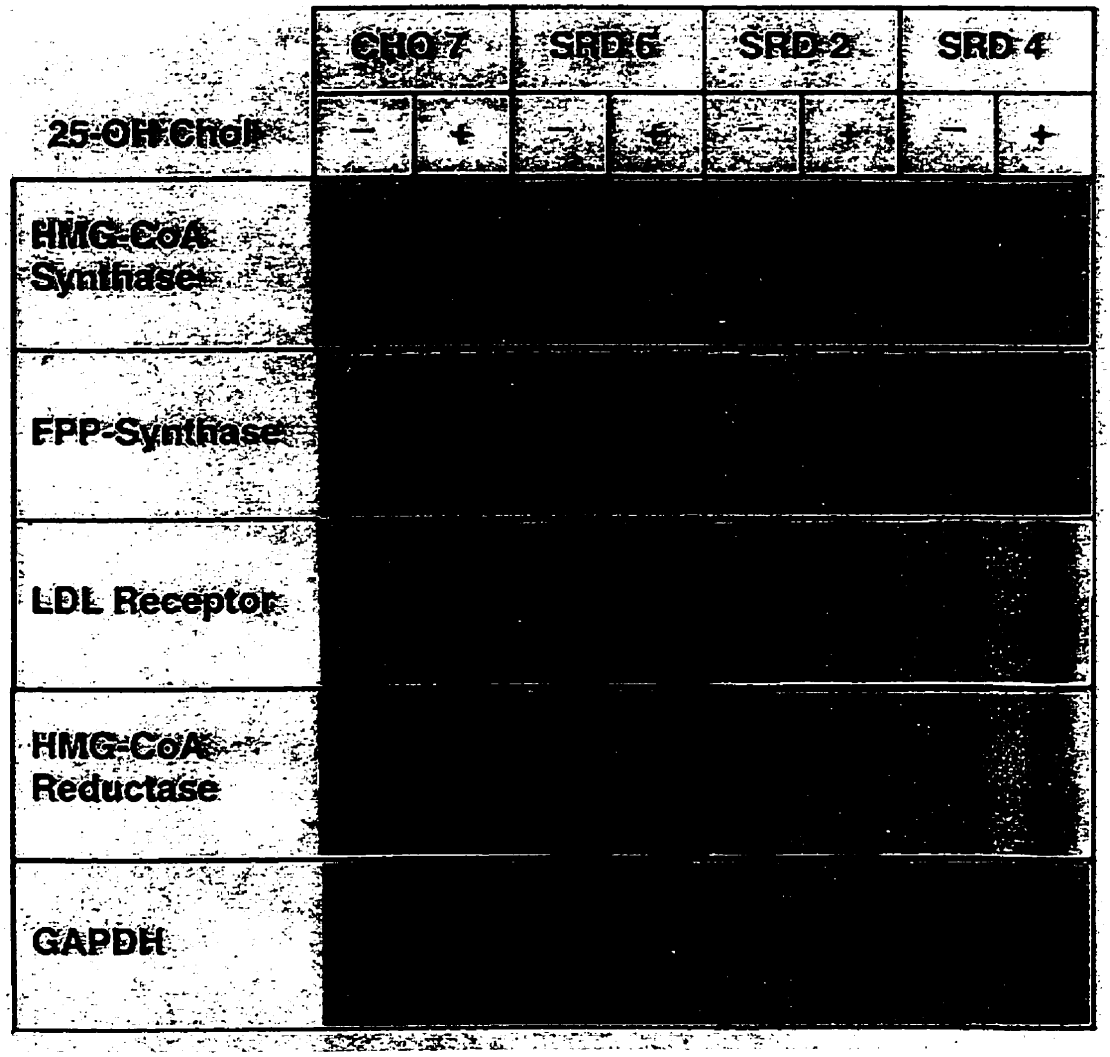
CHO 7
SRD 6
SRD 2
SRD 4
25-OH Chol
−
+
−
+
−
+
−
+
HMG-CoA Synthase
FPP-Synthase
LDL Receptor
HMG-CoA Reductase
GAPDH

Figure 6

and Metherall, 1993), this lack of transcriptional activation in SRD 6 cells was evident by the virtual absence of mRNA for HMG-CoA synthase, farnesyl diphosphate synthase, LDL receptor and HMG-CoA reductase (Figure 6). As seen with the CHO 7 cells, GAPDH gene expression was not affected by this mutation.

The mutation in SRD 2 cells is a genetic recombination that produced a truncated, soluble form of the SREBP-2 protein, which causes constitutive expression of sterol-regulated genes (Yang et al., 1995). This mutant SREBP is missing the transmembrane domains, thus it does not require proteolytic cleavage for activation. In SRD 4 cells, a point mutation in the sterol-cleavage activator protein, which aids in the proteolytic cleavage of the SREBPs, renders cells 25-hydroxycholesterol-resistant with constitutive expression of sterol-regulated genes (Nohturfft et al., 1996). As previously shown (Metherall et al., 1989; Metherall et al., 1991), transcription of HMG-CoA synthase, farnesyl diphosphate synthase, LDL receptor and HMG-CoA reductase was not down regulated by 25-hydroxycholesterol in SRD 2 or SRD 4 cells (Figure 6).

2. Alterations in cholesterol content and synthesis in SRD cells

The total cholesterol content of the SRD cells was measured to determine if this was altered by abnormal expression of sterol-regulated genes (Table 3). CHO 7 cells grown in the presence of cholesterol had slightly elevated cholesterol levels compared to cells grown in cholesterol-free medium. The total cholesterol levels of SRD 6 cells grown without cholesterol were decreased 35% compared to CHO 7 grown under similar conditions. Cholesterol

Table 3. Total cholesterol content of CHO 7 and SRD cell lines.

Cell Line	µg cholesterol/ mg cell protein
CHO 7-	17.0 ± 1.4
CHO 7+	20.1 ± 0.6
SRD 6-	11.1 ± 2.7
SRD 6+	23.2 ± 3.5
SRD 2-	22.3 ± 2.0
SRD 4-	21.5 ± 2.2

Cholesterol mass was assayed in lipid extracts from cells cultured in medium B containing 42 µg cyclodextrin/ml (no cholesterol, CHO 7- SRD 6-, SRD 2-, SRD 4-) or medium B containing 2 µg cholesterol/ml (+ cholesterol, CHO 7+, SRD 6+) for 24 h. Results are the mean and standard deviation of three experiments.

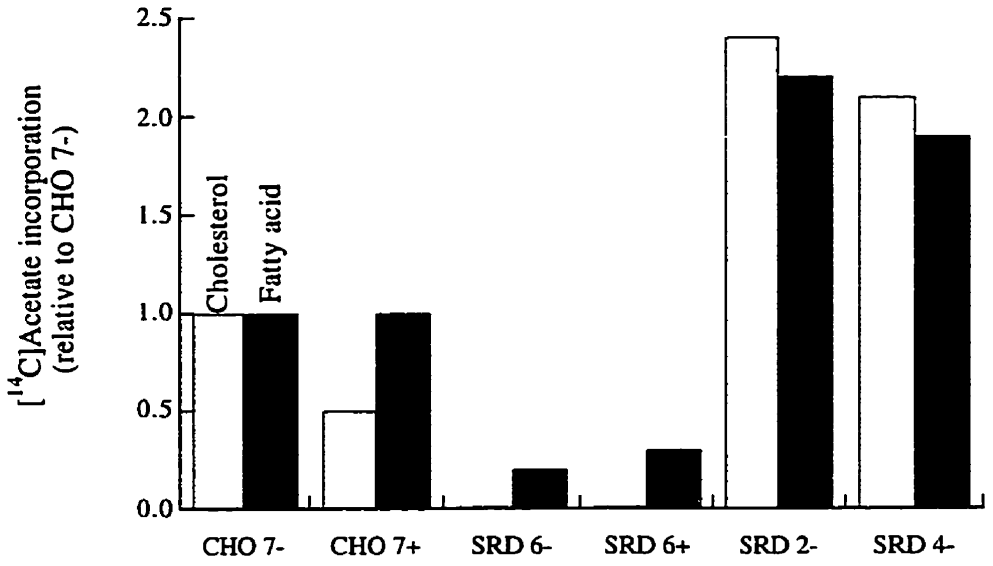

Figure 7. Incorporation of [^{14}C]acetate into cholesterol and fatty acids in CHO 7 and SRD cells. Cells were grown for 24 h in medium B containing 2 μg cholesterol/ml (+) or in medium B containing 42 μg cyclodextrin/ml (-, without cholesterol). Before harvest, cells were incubated for 2 h in medium B containing 50 μM [^{14}C]acetate (2.5 μCi/ml). Radiolabeled cholesterol and fatty acids were isolated and quantified as described in Materials and Methods. Results are from a representative experiment and are expressed relative to the values for CHO 7 -. Similar results were seen in three other experiments.

supplementation of SRD 6 cells normalized the cellular cholesterol levels to that of control CHO 7 cells grown with cholesterol. SRD 2 and SRD 4 cells grown without cholesterol had cholesterol content similar to control cells.

As expected from previous results (Jackson et al., 1996; Evans and Metherall, 1993), mutations in the SRD cell lines altered sterol synthesis, as shown by [^{14}C]acetate incorporation into cholesterol (Figure 7). In CHO 7 cells, addition of cholesterol to the medium caused sterol synthesis to be decreased by 50%. The lack of sterol-regulated gene expression in SRD 6 resulted in a cholesterol synthetic rate of less than 1% of CHO 7 values. SRD 2 and SRD 4cells incorporated 2.5-times more [^{14}C]acetate into cholesterol compared to CHO 7 cells.

Transcription of fatty acid synthase and acetyl-CoA carboxylase, key enzymes involved in fatty acid synthesis, are also regulated by the SREBPs (Magana and Osborne, 1996; Shimano et al., 1996). Thus incorporation of [^{14}C]acetate into fatty acids also varies in the SRD cells (Figure 7). In CHO 7 cells the incorporation of [^{14}C]acetate into fatty acid was not affected by the addition of cholesterol to the media. However, [^{14}C]acetate incorporation into fatty acids in SRD 6 cells was decreased 70-80% compared to CHO 7 cells. Conversely, synthesis of [^{14}C]fatty acids in SRD 2 and SRD 4 cells was increased 2-fold.

3. Regulation of ACAT activity

The activity of ACAT was also affected by mutations in the SRD cell lines (Figure 8). CHO 7 cells, grown without cholesterol and treated with 25-

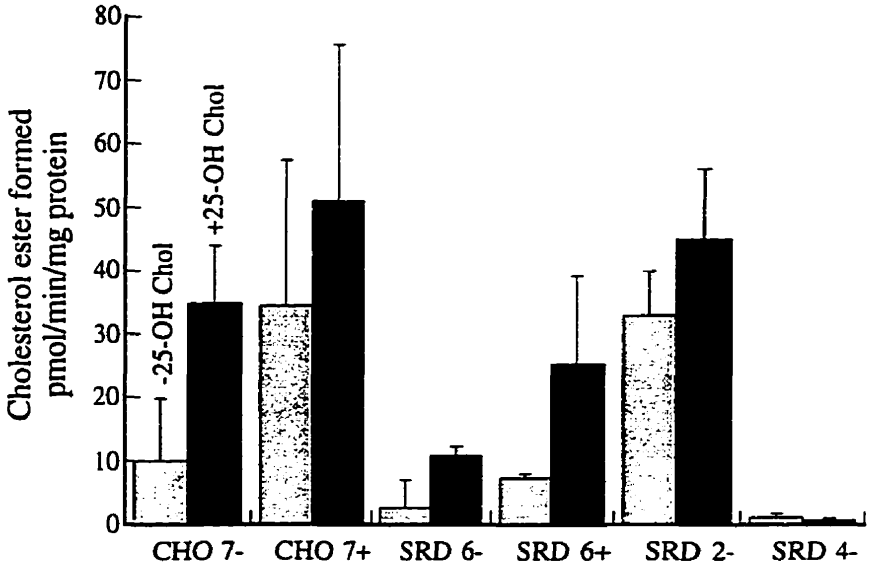

Figure 8. ACAT activity in CHO 7 and SRD cells. Cells were cultured with and without cholesterol for 24 h as described in Table 3. For the last 4 h of the treatment, cells were incubated with 25-hydroxycholesterol (2.5 μg/ml, black bars) or solvent control (ethanol, gray bars) and pulsed with 100 μM [^{3}H]oleate for 60 min immediately prior to harvesting. [^{3}H]Oleate incorporation into cholesterol ester was quantitated as described in Materials and Methods. Results are the mean and range of two or three separate experiments.

hydroxycholesterol displayed a 3- to 4-fold increase in [^{3}H]cholesteryl ester formation compared to non-oxysterol treated cells. Growth of CHO 7 cells in cholesterol for 24 h caused an increase in the basal ACAT activity and a relative decrease in the fold stimulation by 25-hydroxycholesterol. SRD 6 cells, grown either in the absence and presence of cholesterol, exhibited decreased basal ACAT activity compared to CHO 7 cells, but ACAT activity responded 3-fold to 25-hydroxycholesterol treatment. Due to their higher rate of cholesterol synthesis, SRD 2 cells had increased basal ACAT activity compared to controls, and the response to 25-hydroxycholesterol was only slight compared to non-oxysterol treated cells. SRD 4 cells had no cholesterol esterification activity in the presence or absence of 25-hydroxycholesterol due to a point mutation in the ACAT gene (Cao et al., 1996). These results are similar to those previously reported by Metherall et al. (1991) and Evans and Metherall (1993).

4. Growth characteristics of SRD 6 cells

Proliferation of cholesterol auxotrophic SRD 6 cells requires the presence of cholesterol in the culture medium. SRD 6 cells express very low LDL receptor levels (Evans and Metherall, 1993), thus cholesterol must be added in a non-lipoprotein form. Cholesterol delivery to SRD 6 cells either as cholesterol dissolved in ethanol or a water-soluble complex with β-methyl cyclodextrin was assessed. We compared the growth rates of SRD 6 cells grown in media containing these forms of cholesterol (Figure 9). SRD 6 cells grew better with cholesterol complexed to cyclodextrin (Figure 9, panel A) compared to cholesterol dissolved in ethanol (Figure 9, panel B). β-Methyl cyclodextrin alone

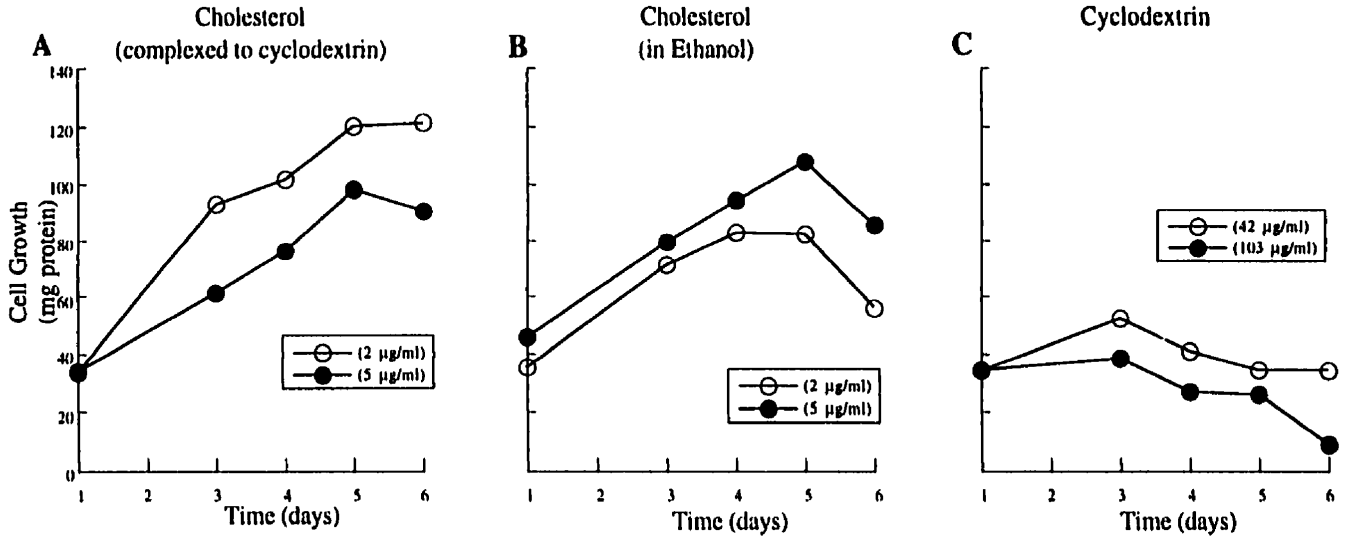

Figure 9. Growth rates of SRD 6 cells with cholesterol supplementation. SRD 6 cells were grown in medium B containing 1 mM mevalonate and either cholesterol complexed to cyclodextrin (panel A, ○ = 2 µg cholesterol/ml, 42 µg cyclodextrin/ml, ● = 5 µg cholesterol/ml, 103 µg cyclodextrin/ml), cholesterol dissolved in ethanol (panel B, ○ = 2 µg/ml, ● = 5 µg/ml), or cyclodextrin alone (panel C, ○ = 42 µg/ml, ● = 103 µg/ml). Cells were harvested in 0.1 N NaOH and protein was determined (Lowry et al. 1951). The results are the average of 2 dishes from a representative experiment.

Figure 10. DNA and protein synthesis in CHO 7 and SRD 6 cells. CHO 7 (□, ■) and SRD 6 (○, ●) cells were incubated in medium B with (solid symbols) or without cholesterol (open symbols) for 24 h as described in the legend for Figure 8. For DNA synthesis (panel A), cells were incubated for 2 or 4 h in medium B containing the above additions and 1 μCi [^{14}C]thymidine/ml. Label incorporation was assessed as described in Materials and Methods. Results are the mean and standard deviation of four separate experiments. For protein synthesis (panel B) cells were incubated in methionine and cystine-free medium B with the additions described above for 1 h [^{35}S]methionine (2 μCi/ml) was added and cells were harvested at 2 or 4 h. Results are the average and range for two experiments.

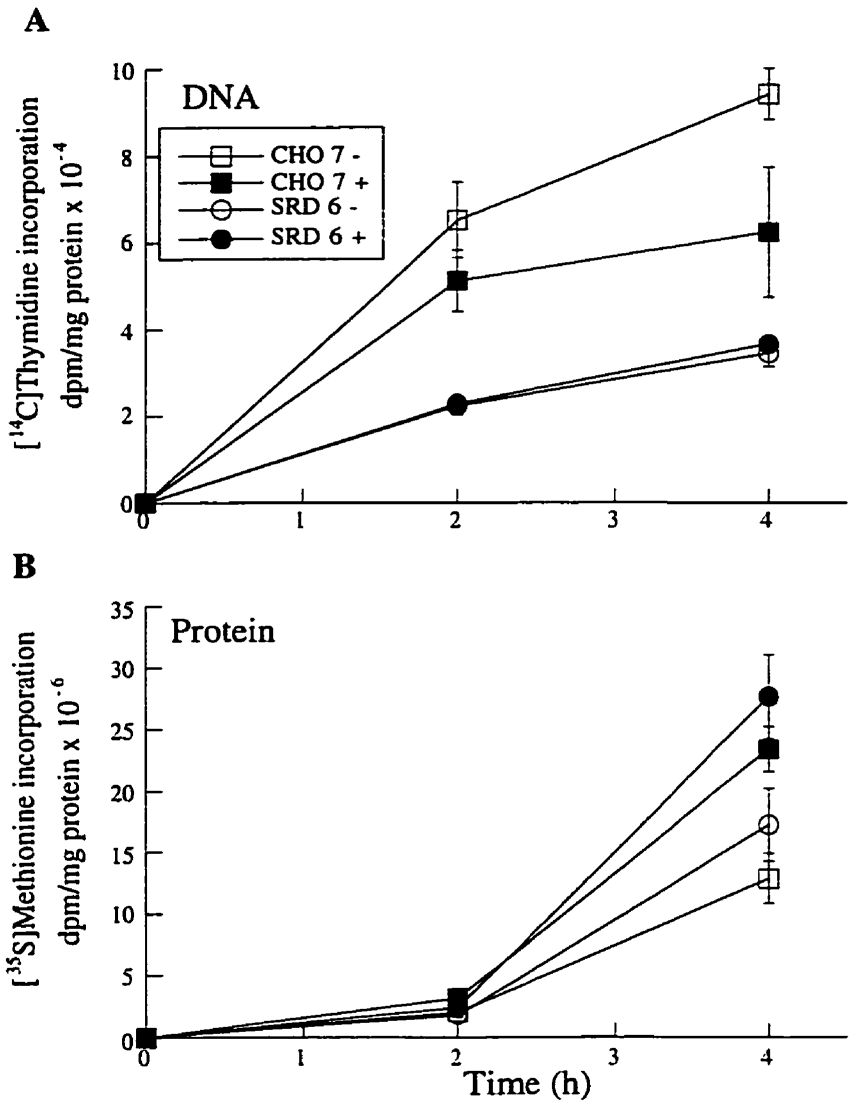
A
DNA
CHO 7 -
CHO 7 +
SRD 6 -
SRD 6 +
[14C]Thymidine incorporation
dpm/mg protein x 10-4
B
Protein
[35S]Methionine incorporation
dpm/mg protein x 10-6
Time (h)

Figure 10

did not support SRD 6 cell growth (Figure 9, panel C). Cyclodextrin was also slightly toxic to cells as shown in the cholesterol supplement cells; SRD 6 cells cultured in 5 μg cholesterol complexed to cyclodextrin/ml grew slower than those grown in 2 μg/ml. When cholesterol dissolved in ethanol was used, the cellular growth rate was increased with 5 μg/ml compared to 2 μg/ml, but cell growth seemed to decrease at days 5 and 6. Since SRD 6 cell growth was optimal with 2 μg cholesterol/ml complexed to cyclodextrin, this concentration was used for maintaining stock cultures and in experimental procedures. This cholesterol preparation contained 42 μg cyclodextrin/ml and non-sterol controls received this amount of cyclodextrin.

The cholesterol auxotrophy of the SRD 6 cell line decreased its growth rate compared to that of CHO 7 cells, as shown by decreased [^{14}C]thymidine incorporation into DNA (Figure 10, panel A). The incorporation rate of thymidine in the SRD 6 cells was decreased 50-70% compared to the CHO 7 cells, indicating a slower rate of cell division. Addition of cholesterol to SRD 6 cells did not alter DNA synthesis. In CHO 7 cells, however, growth without cholesterol caused a slight increase in [^{14}C]thymidine incorporation compared to cells grown with cholesterol. The protein synthetic rates, measured by [^{35}S]methionine incorporation (panel B) were similar in both cell lines and treatment with cholesterol increased [^{35}S]methionine incorporation by 2-fold at 4 h.

B. Regulation of phosphatidylcholine synthesis in SRD cells

1. PtdCho biosynthesis in SRD cells

PtdCho is the major phospholipid in cells, comprising up to 50% of the total phospholipid mass, and alterations in PtdCho metabolism are important for the maintenance of a proper phospholipid-to-cholesterol ratio. SRD cells were used to study the effect of altered cholesterol metabolism, due to SREBP mutations, on PtdCho biosynthesis. CHO 7 and SRD cells were grown in the absence and presence of cholesterol for 24 h prior to labeling with [^{3}H]choline for 2 and 4 h (Figure 11). The incorporation of radiolabel into PtdCho in SRD 6 cells grown without cholesterol was reduced by 70% and 50% at 2 and 4 h, respectively, compared to CHO 7 cells. [^{3}H]Choline incorporation into SM was also reduced, probably as a result of the lower specific radioactivity of its precursor, PtdCho. Addition of cholesterol to the medium for 24 h did not appreciably affect labeling of PtdCho or SM in either SRD 6 or CHO 7 cells. In SRD 2 and SRD 4 cells, labeling of PtdCho and SM was similar to CHO 7 cells.

The PtdCho content of cells is usually regulated within a narrow range, probably the result of coordinate synthetic and degradation rates (Pelech and Vance, 1989; Walkey et al., 1994; Terce et al., 1991; Tijburg et al., 1991). Phospholipid mass was measured in CHO 7 and SRD 6 cells to determine if reduced PtdCho synthesis in SRD 6 cells was reflected by reduced PtdCho mass (Figure 12A). SRD 6 cells depleted of cholesterol for 24 h exhibited a significant 8% reduction in PtdCho mass compared to CHO 7 cells. Incubation with cholesterol increased PtdCho mass such that it was no longer significantly

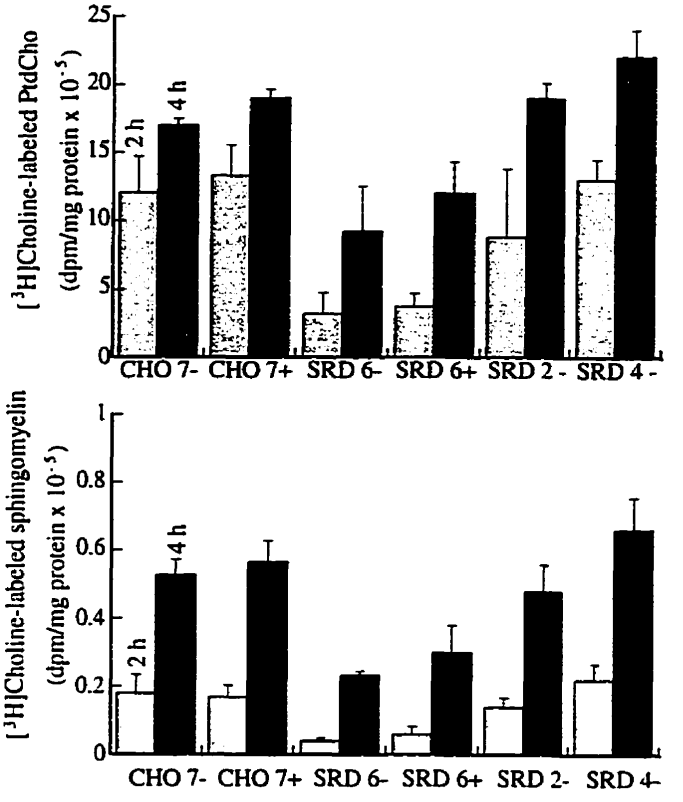

Figure 11. Incorporation of [^{3}H]choline into PtdCho and SM in CHO 7 and SRD cells. Cells were grown in medium B (42 μg cyclodextrin/ml, - cholesterol) or medium C (2 μg cholesterol/ml complexed with cyclodextrin and 1 mM mevalonate, + cholesterol) for 24 h. The medium was changed to choline-free medium B ± cholesterol, and after 1 h cells received the same media containing 2 μCi [^{3}H]choline/ml for 2 (gray bars) or 4 h (solid bars). Cells were harvested and the amount of labeled PtdCho and SM was quantitated as described in Materials and Methods. Results are the mean and SEM for three separate experiments.

Figure 12. Phospholipid composition of CHO 7 and SRD cells. Panel A, CHO 7 and SRD 6 cells were cultured in medium B with cholesterol (+) or with cyclodextrin (-) for 24 h as described in the legend to Figure 8. Panel B, CHO 7, SRD 2 and SRD 4 cells were cultured in medium B (without cholesterol or cyclodextrin) for 24 h. Cells were harvested, and phospholipids were isolated and quantitated as described in Materials and Methods. Insert, total phospholipid:protein ratio in the 4 cell lines. Results are the mean and standard deviation of four separate experiments. Significance was determined using two-tailed *t*-test compared to CHO 7 cells incubated without cholesterol (*=$P<0.05$, **=$P<0.025$).

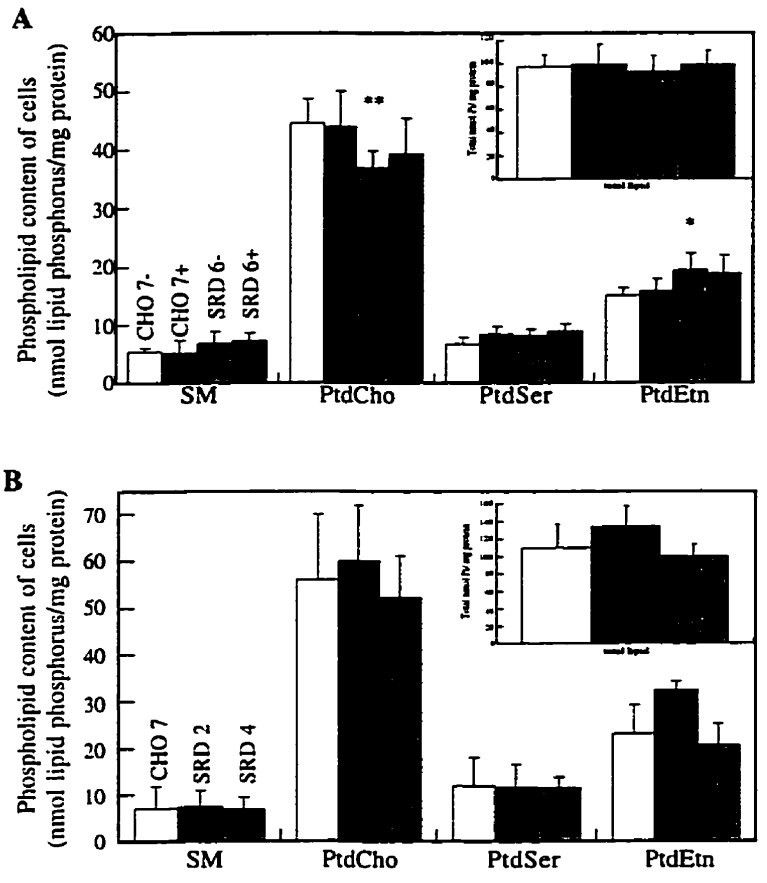
A
Phospholipid content of cells
(nmol lipid phosphorus/mg protein)
60
50
40
30
20
10
0
CHO 7-
CHO 7+
SRD 6-
SRD 6+
**
*
SM
PtdCho
PtdSer
PtdEtn
B
Phospholipid content of cells
(nmol lipid phosphorus/mg protein)
70
60
50
40
30
20
10
0
CHO 7
SRD 2
SRD 4
SM
PtdCho
PtdSer
PtdEtn

Figure 12

different from CHO 7 cells grown under the same conditions. The reduced mass of PtdCho in SRD 6 cells grown without cholesterol was compensated for by slight elevations in the other phospholipids, but this increase was only significant with PtdEtn. This compensatory increase in the other phospholipids ensured that the total phospholipid:protein ratio in SRD 6 and CHO 7 cells was similar, regardless of cholesterol treatment (Figure 12A, insert). The total phospholipid:protein ratios in SRD 2 and SRD 4 cells were also identical to that of the CHO 7 cells (Figure 12B, insert). The phospholipid composition of these cells were similar to controls (Figure 12B).

To determine which step in the CDP-choline pathway was inhibited in SRD 6 cells, the distribution of the water-soluble metabolites was examined after incubation with [^{3}H]choline for 2 and 4 h (Figure 13). In SRD 6 cells grown in the absence of cholesterol, the amount of label present in the precursor fractions (choline, Pcho and CDP-choline) was elevated compared to CHO 7 cells. The most significant difference, however, was a >10-fold increase in [^{3}H]Pcho levels. The total amount of [^{3}H]choline incorporated into both water-soluble and lipid fractions was identical in both CHO 7 and SRD 6 cells, and cholesterol treatment for 24 h did not alter the metabolic profiles for either cell line. From the water-soluble metabolites, the rate of degradation of PtdCho can be determined indirectly by the rate of [^{3}H]choline incorporation into GroPcho (Figure 13), which was similar in SRD 6 and CHO 7 cells.

The water-soluble choline metabolite profiles of SRD 2 and SRD 4 cells were also different compared to CHO 7 cells (Figure 13). In both SRD 2 and

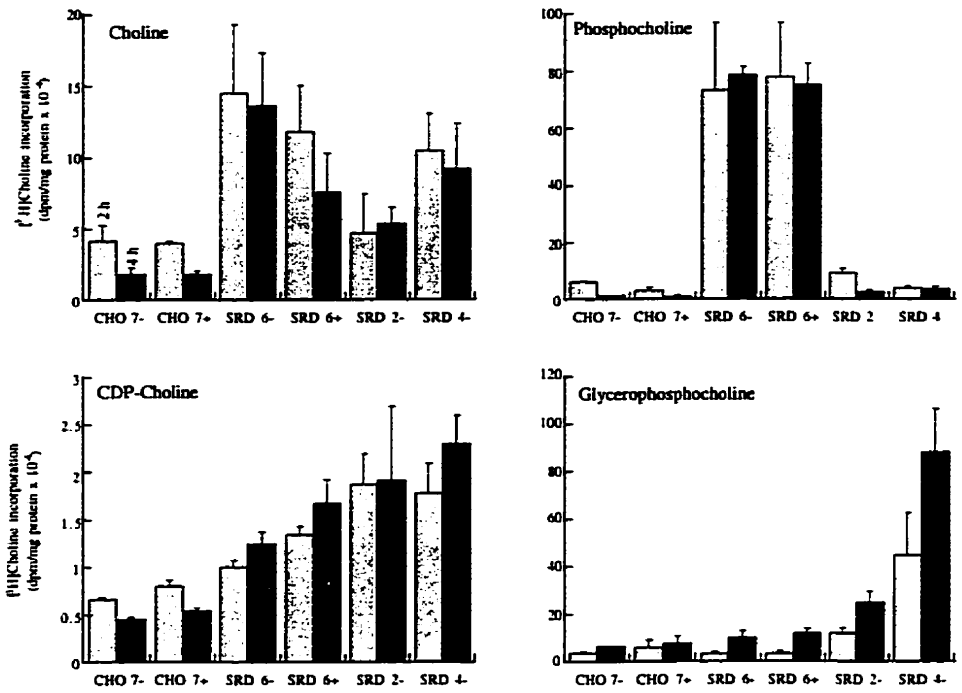

Figure 13. [^{3}H]choline-labeled metabolites in CHO 7 and SRD cells. Cells were grown in medium B with (+) or without (-) cholesterol and labeled with [^{3}H]choline for 2 h (gray bars) or 4 h (solid bars) as described in the legend to Figure 11. Choline metabolites were extracted, separated by TLC, and quantified as described in Materials and Methods. Results are the mean and SEM for three separate experiments.

SRD 4 cells there was increased cellular [^{3}H]choline, and [^{3}H]CDP-choline, compared to CHO 7 controls. The most notable difference, however, was in the [^{3}H]GroPcho fraction which was increased by 12- to 14-fold in SRD 4 cells and 3- to 4-fold in SRD 2 cells. Total [^{3}H]choline incorporated into the lipid and water-soluble metabolites was similar in SRD 2 and CHO 7 cells, but increased 1.5- to 1.8-fold in SRD 4 cells at 2 and 4 h, respectively.

To further demonstrate abnormal Pcho metabolism in SRD 6 cells, the turnover of [^{3}H]choline metabolites in SRD 6 and CHO 7 cells was examined by pulse-chase experiments (Figure 14). [^{3}H]Choline levels were rapidly reduced in both cell lines, and 30 min after the removal of isotope the level had reached a minimum. In CHO 7 cells, [^{3}H]Pcho was reduced to very low levels (70% of initial values) at the end of the 2 h chase period. In contrast, [^{3}H]Pcho levels in SRD 6 cells was only decreased by 40%, but the loss of total [^{3}H]Pcho in SRD 6 cells was 4-fold greater than controls. As with the labeling studies shown in Figure 13, comparison of [^{3}H]choline incorporation into PtdCho and Pcho at the start of the pulse indicated that accumulation of radioactivity in Pcho largely accounts for the reduced incorporation into PtdCho in SRD 6 cells. The excess [^{3}H]Pcho in SRD 6 cells appears to be slowly incorporated into [^{3}H]PtdCho, which increased steadily during the chase period. In CHO 7 cells, [^{3}H]PtdCho levels decreased during the chase period. Also, [^{3}H]GroPcho production was reduced in SRD 6 cells, indicating a delay in PtdCho degradation compared to CHO 7 cells. Release of radioactivity into the medium by SRD 6 and CHO 7 cells was similar, with the exception of a slight increase for SRD 6 cells grown

Figure 14. Turnover of [^{3}H]choline-labeled metabolites in SRD 6 and CHO 7 cells. Cells were grown with (closed symbols) or without cholesterol (open symbols) as described in Figure 11. SRD 6 (□, ■) and CHO 7 (○, ●) cells were pulse-labeled for 1 h with 2 μCi [^{3}H]choline/ml in medium B followed by medium B without isotope. At the indicated times, cells were harvested and choline metabolites and PtdCho were quantitated. Results are the mean and SEM of three experiments.

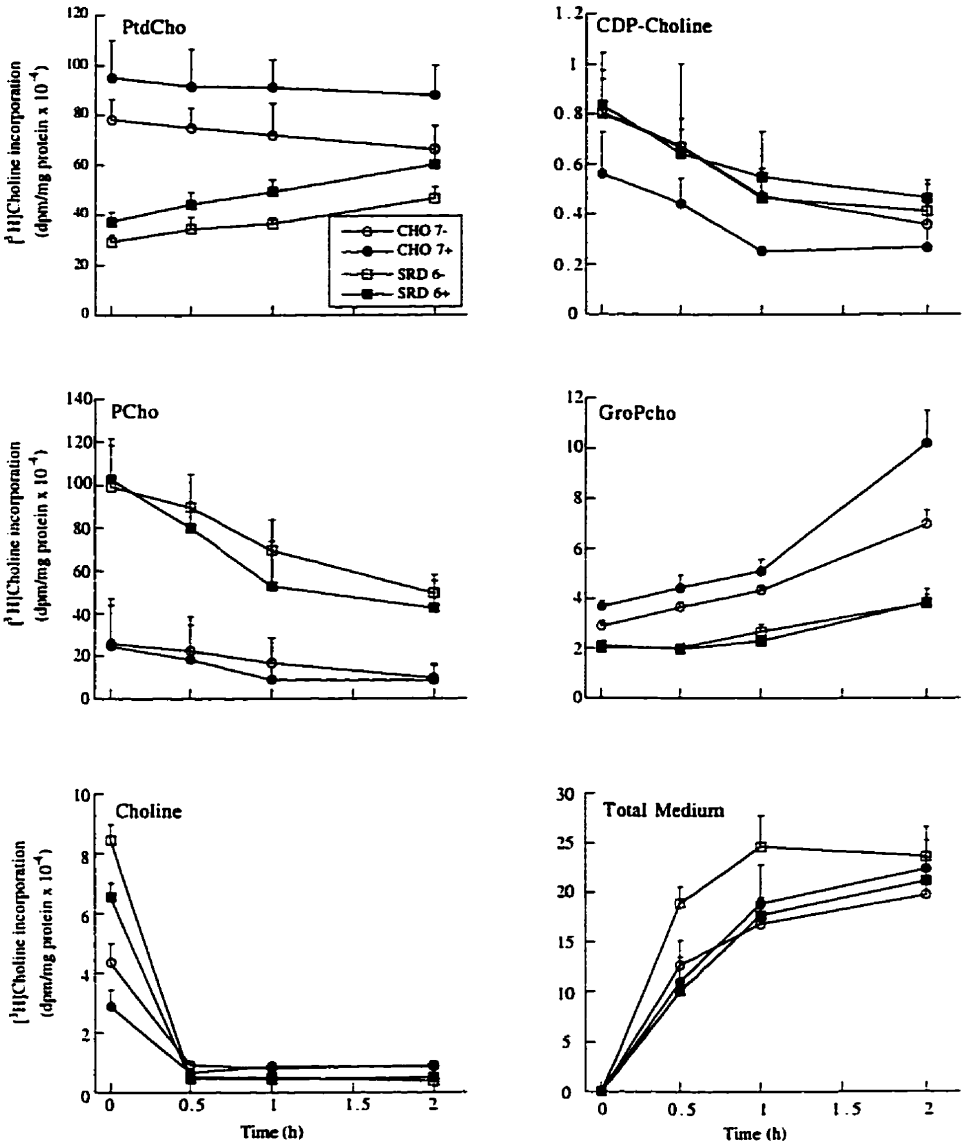
PtdCho
CDP-Choline
PCho
GroPcho
Choline
Total Medium
[³H]Choline incorporation (dpm/mg protein x 10⁻⁴)
Time (h)
CHO 7-
CHO 7+
SRD 6-
SRD 6+

Figure 14

without cholesterol. [^{3}H]Choline accounted for 80% and 70% of medium radioactivity for CHO 7 and SRD 6 cells, respectively, with the remainder found in [^{3}H]Pcho.

Consistent with the elevated labeling of Pcho and slowed conversion to PtdCho, Pcho mass in SRD 6 cells grown in the presence or absence of cholesterol was increased relative to CHO 7 cells (Table 4). In SRD 6 cells, Pcho mass was increased 1.7-fold compared to CHO 7 cells. The Pcho mass in SRD 2 and SRD 4 cells was not significantly different than CHO 7 controls.

2. Regulation and distribution of cytidylyltransferase in SRD cells

Conversion of Pcho to CDP-choline, catalyzed by the rate-limiting enzyme CTP:phosphocholine cytidylyltransferase, appeared to be compromised in SRD 6 cells. To determine if the activities of CT, or the other two enzymes of the CDP-choline pathway, were altered in the SRD cell lines, the *in vitro* activities for these three enzymes were compared to CHO 7 cells. The activities of choline kinase and cholinephosphotransferase were not significantly different in any of the SRD mutants compared to CHO 7 cells (Table 5). Cytosolic CT activity assayed in the presence or absence lipid activator (oleate/PtdCho vesicles) was increased 2- and 3-fold, respectively, in SRD 6 cells grown without cholesterol (Table 6). CT activity was also elevated 2-fold in cytosol from SRD 6 cells grown with cholesterol, but this result was only significant when assayed in the presence of lipid activators. In contrast, the total membrane fractions from SRD 6 and CHO 7 cells had similar CT activities regardless of the presence or

Table 4. Phosphocholine mass in CHO 7 and SRD cells.

Cell line	Phosphocholine
	nmol phosphocholine/mg cell protein
CHO 7-	6.9 ± 1.2
CHO 7+	7.2 ± 0.7
SRD 6-	12.6 ± 1.7
SRD 6+	11.9 ± 1.9
SRD 2-	4.6 ± 0.5
SRD 4-	4.6 ± 0.7

CHO 7 and SRD 6 cells were grown with (+) or without (-) cholesterol for 24 h as described in Figure 11. Phosphocholine was isolated and quantitated as described in Materials and Methods. Results are the mean and SEM of three separate experiments.

Table 5. Activity of choline kinase and choline phosphotransferase in CHO 7 and SRD cells

	Choline kinase	Choline phosphotransferase
	nmol/min/mg protein	
CHO 7-	0.40 ± 0.08	0.56 ± 0.08
CHO 7+	0.37 ± 0.05	0.57 ± 0.08
SRD 6-	0.35 ± 0.05	0.54 ± 0.05
SRD 6+	0.44 ± 0.09	0.64 ± 0.14
SRD 2-	0.31 ± 0.10	0.65 ± 0.09
SRD 4-	0.29 ± 0.11	0.79 ± 0.25

Enzyme activities were assayed in cytosol or membranes isolated from cells cultured in medium B containing 42 µg cyclodextrin/ml (- cholesterol), or medium C (+ cholesterol) for 24 h. Results are the mean and SEM of three to eight separate experiments.

Table 6. Activity of CTP:phosphocholine cytidylyltransferase in CHO 7 and SRD cells

	CTP:phosphocholine cytidylyltransferase			
	cytosol -oleate/PtdCho	cytosol + oleate/PtdCho	membrane -oleate/PtdCho	membrane + oleate/PtdCho
	nmol/min/mg protein			
CHO 7 -	0.44 ± 0.08	1.28 ± 0.11	0.85 ± 0.10	1.06±0.26
CHO 7 +	0.40 ± 0.05	1.15 ± 0.14	1.02 ± 0.10	1.14±0.12
SRD 6-	1.24 ± 0.32[b]	2.66 ± 0.34[a]	1.16 ± 0.13	1.42±0.16
SRD 6+	0.70 + 0.13	2.17 + 0.32[b]	1.30 + 0.12	1.46±0.28
SRD 2-	0.54 ± 0.10	1.45 ± 0.24	0.90 ±0.11	N.D.
SRD 4-	0.25 ± 0.06	0.94 ± 0.15	1.03 ± 0.25	N.D.

Enzyme activities were assayed in cytosol or membranes, isolated from cells cultured in medium B with (+) or without (-) cholesterol for 24 h, in the presence of absence of 0.1 mM oleate/PtdCho vesicles. Results are the mean and SEM for three to eight separate experiments. Significance was determined using a two-tailed *t*-test and compared to the corresponding activity for CHO 7 cells cultured without cholesterol for 24 h ([a], $p<0.005$; [b], $p<0.05$) N.D. = not determined

absence of PtdCho/oleate vesicles. CT activity in SRD 2 cells, both in the cytosol and membrane fractions, was similar to those in CHO 7 cells. SRD 4 cells had a slight shift of CT activity from cytosol to membranes, but this result was not statistically significant.

Immunoblot analysis of CT in total cell lysates, cytosol and membrane fractions is shown in Figure 15. It is evident that immunoreactive enzyme protein in detergent extracts of SRD 6 cells (Figure 15, panel A) was increased approximately 2-fold compared to similar extracts from CHO 7 cells. Consistent with results for CT activity, immunoreactive enzyme mass was increased in SRD 6 cell cytosol (Figure 15, panel B) but not in the membrane fraction (Figure 15, panel C). It was consistently observed that CT was resolved into 2 or 3 bands due to altered mobility in SDS-PAGE, presumably as the result of extensive phosphorylation (Watkins and Kent, 1991). The distribution of phosphorylated isoforms of CT was similar between SRD 6 and CHO 7 cells in total extracts and membranes. However, cytosolic CT in SRD 6 cells, appeared to have less of the low molecular weight (dephosphorylated) isoform compared to controls. SRD 2 cells showed similar immunoreactive enzyme mass and banding patterns seen for CHO 7 cells in all fractions investigated. SRD 4 cells seemed to contain less immunoreactive CT in both the total lysate and cytosolic fractions. CT present within the cytosol of SRD 4 cells was predominately in the low molecular weight form. As with SRD 2 and 6 cells, the amount of immunoreactive CT present within the membranes from SRD 4 cells was similar to that in CHO 7 cells.

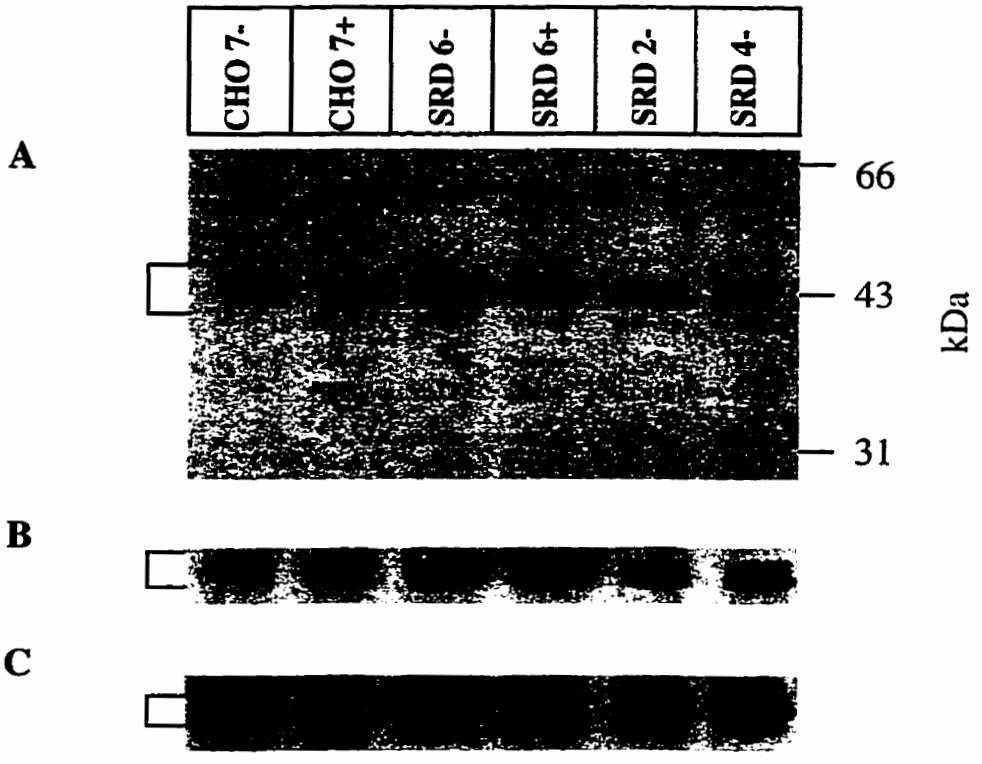

Figure 15. Immunoblot analysis of CT in CHO 7 and SRD cells. Cells were grown in medium B with or without cholesterol for 24 h as described in the legend to Figure 11. Total cell lysates (A), cytosol (B), and membranes (C) were prepared and 15 μg of protein from each fraction was subjected to 10% SDS-PAGE and immunoblotting as described in Materials and Methods. Similar results were seen in three other experiments. The position of CT on the immunoblot is indicated by brackets.

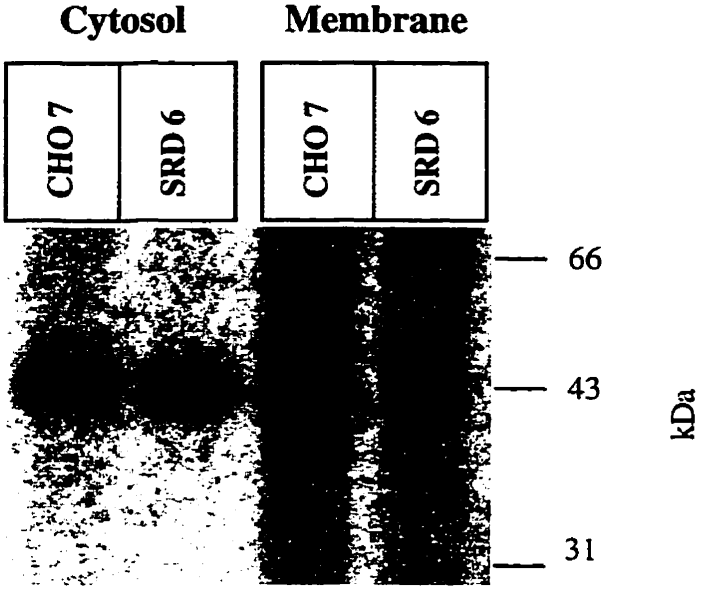

Figure 16. Phosphorylation of CT in SRD 6 and CHO 7 cells. CT in SRD 6 and CHO 7 cells (grown for 24 h in medium B, without cholesterol) was labeled with [^{32}P]phosphate (25 μCi/ml for 12 h) and immunoprecipitated from 50 μg of cytosol and membrane protein as described in Materials and Methods. Immunoprecipitates were separated by 10% SDS-PAGE and the dried gel was exposed to Kodak BioMax film for 48 h at -70°C. Similar results were seen in two other experiments.

To determine whether CT phosphorylation changes in SRD 6 cells could be related to the increased cytosolic distribution of the enzyme, the [^{32}P]phosphate content of CT in SRD 6 and CHO 7 cells grown without cholesterol for 24 h was examined by immunoprecipitation (Figure 16). [^{32}P]Phosphate incorporation into cytosolic CT was similar in control and SRD 6 cells. However, CT mass was increased in SRD 6 cytosol (Figure 15), indicating that cytosolic CT from these mutant cells was slightly dephosphorylated relative to controls. Phosphorylation of the membrane-bound form of the enzyme was reduced in both cell lines compared to the soluble enzyme, and CT from SRD 6 membranes also appeared to be slightly dephosphorylated relative to controls.

CT mRNA levels were quantitated in SRD cells and CHO 7 cells by S1 nuclease protection analysis to determine if changes in mRNA levels could account for the changes in CT expression in SRD 6 cells (Figure 17). The results showed that CT mRNA levels in SRD 6 cells were not significantly different than CHO 7 cells, and that cholesterol supplementation did not effect CT gene transcription. However, CT mRNA expression in SRD 4 cells was decreased by 30% compared to CHO 7 cells. mRNA levels in SRD 2 cells were not significantly different than CHO 7 cells.

The difference in PtdCho biosynthesis in the SRD 6 cells could be due altered kinetic properties of CT. *In vitro* enzyme assays, shown in Table 6, were performed under optimal conditions and subtle differences in enzyme kinetics may not be apparent. Kinetic constants (K_m and V_{max}) for both Pcho and CTP, were determined from linear double-reciprocal plots of CT activity in membrane

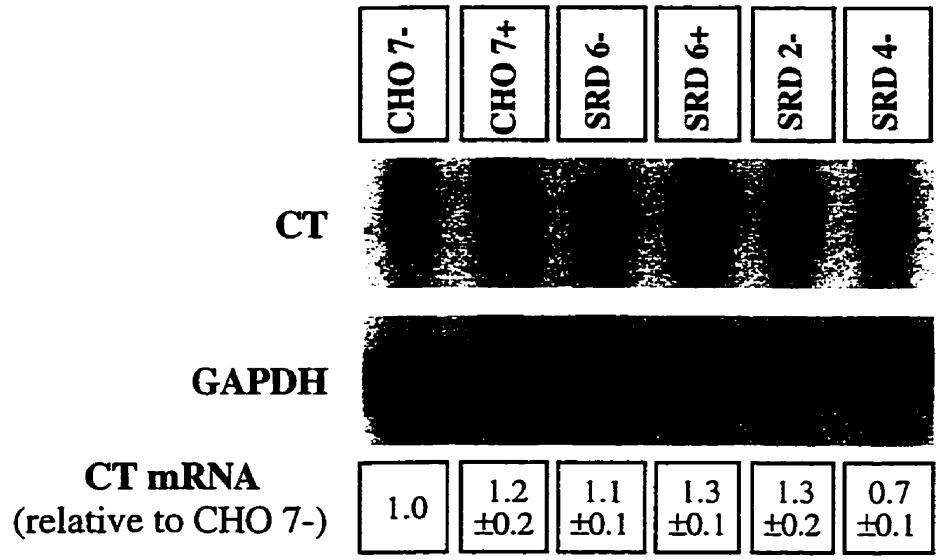

Figure 17. CT mRNA expression in CHO 7 and SRD cell lines. Cells were grown with (+) or without (-) cholesterol for 24 h as described in the legend to Figure 11. The amount of RNA assayed was 30 μg for CT and 10 μg for GAPDH, and 100,000 cpm of probe was used for both reactions. CT mRNA levels were normalized to GAPDH mRNA and expressed relative to CHO 7 cells grown in the absence of cholesterol (CHO 7 -). Results are the mean and SEM for three separate experiments.

Table 7. Kinetic Constants for CTP:phosphocholine cytidylyltransferase from CHO 7 and SRD 6 cells.

	Phosphocholine		CTP	
	K_m *(mM)*	V_{max} *(nmol/min/mg protein)*	K_m *(mM)*	V_{max} *(nmol/min/mg protein))*
CHO 7	0.19 ± 0.04	1.69 ± 0.12	0.77 ± 0.33	1.45 ± 0.41
SRD 6	0.25 ± 0.06	1.18 ± 0.13 [a]	0.60 ± 0.20	1.12 ± 0.50

Kinetic constants were determined by assaying enzyme activities from membranes of CHO 7 and SRD 6 cells grown in medium B (without cholesterol) for 24 h, with varying concentrations of either phosphocholine or CTP at fixed CTP (3 mM) and phosphocholine (1.5 mM), respectively, in the absence of lipid activators. Results are the mean and SEM of three separate experiments. Significance was determined using a two-tailed *t*-test and compared to the corresponding value for CHO 7 cells ([a]$P<0.05$).

fractions isolated from CHO 7 and SRD 6 cells grown without cholesterol for 24 h (Table 7). The K_m for both substrates were similar for CT from CHO 7 and SRD 6 cells. The CT V_{max} for Pcho in SRD 6 cells, however, was significantly decreased by 30% compared to CHO 7 controls. The V_{max} for CTP was also reduced for SRD 6 CT, but this reduction was not statistically significant.

3. PtdCho synthesis and CT translocation in oleate-treated SRD 6 and CHO 7 cells

In vitro and *in vivo* studies have shown that CT translocates to membranes in the presence of various lipid activators (Tronchere et al., 1994; Cornell and Vance, 1987a) such as long-chain fatty acids. To determine whether CT in SRD 6 cells responds to this stimulus, cells were treated with oleate and [^{3}H]choline incorporation into PtdCho, SM and the soluble metabolites was measured (Figure 18). Treatment of CHO 7 cells with 150 µM oleate caused a 2- to 3-fold increase in [^{3}H]PtdCho synthesis at 1 and 2 h after [^{3}H]choline addition, and markedly reduced the lag in PtdCho synthesis observed in untreated cells. As shown previously in Figure 11, incorporation of label into PtdCho was significantly reduced in untreated SRD 6 cells compared to controls. Oleate treatment stimulated synthesis in SRD 6 cells such that by 4h [^{3}H]choline incorporation into PtdCho was similar to untreated CHO 7 cells. Oleate-treated CHO 7 and SRD 6 cells had significantly reduced [^{3}H]Pcho compared to their untreated counterpart at 1 and 2 h of the [^{3}H]choline pulse. By 4 h, the level of labeled Pcho in oleate-treated SRD 6 cells was similar to that in oleate-treated and untreated CHO 7 cells. Enhanced conversion of Pcho to CDP-choline in

Figure 18. Stimulation of PtdCho synthesis by oleate in SRD 6 and CHO 7 cells. CHO 7 (○,●) and SRD 6 (□, ■) cells were grown for 24 h in medium B (without cholesterol) prior to receiving choline-free medium B with (+, closed symbols) or without (-,open symbols) 150 μM oleate complexed to BSA. After 1 h, [^{3}H]choline (2 μCi/ml) was added directly to each dish and at indicated times cells were harvested for quantitation of PtdCho, SM and the soluble choline metabolites. Results are the average and SEM of four determinations from 2 separate experiments.

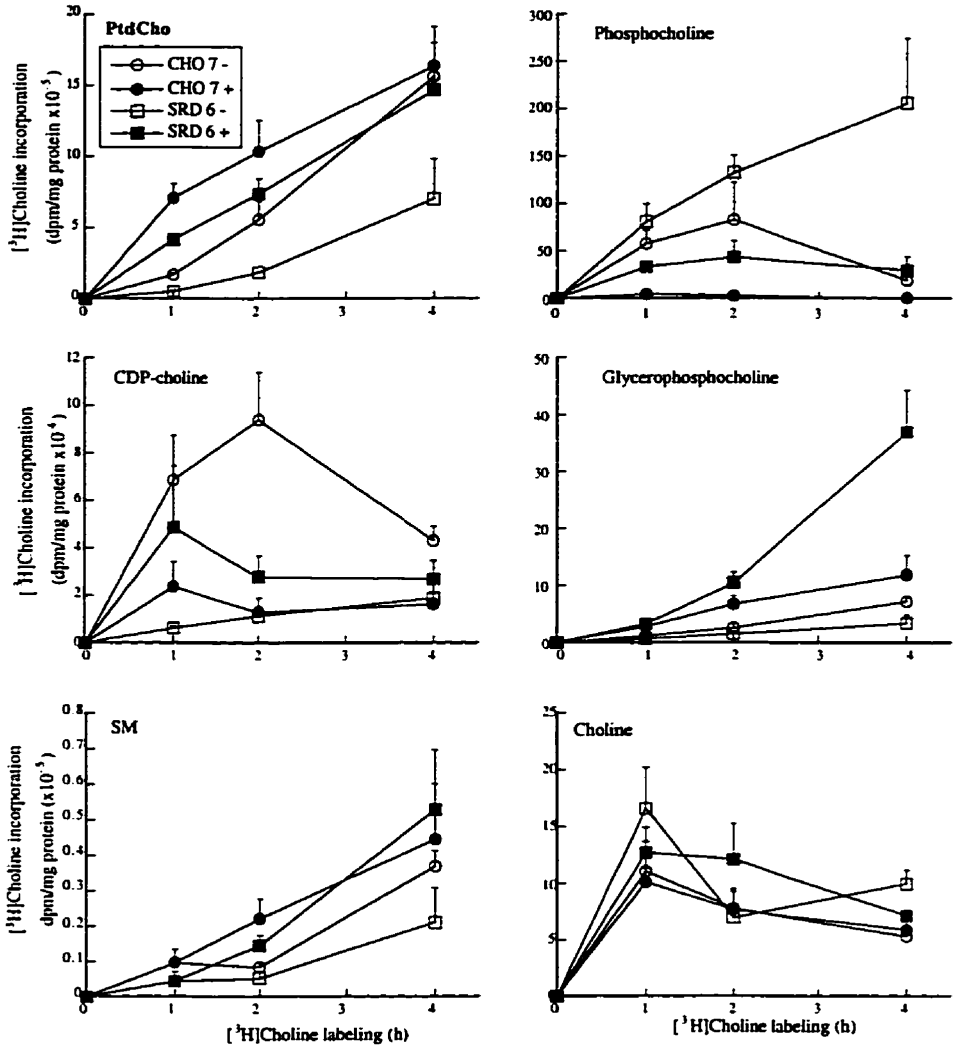
PtdCho
CHO 7 -
CHO 7 +
SRD 6 -
SRD 6 +
Phosphocholine
CDP-choline
Glycerophosphocholine
SM
Choline
[³H]Choline incorporation (dpm/mg protein x10⁻³)
[³H]Choline incorporation (dpm/mg protein x10⁻⁴)
[³H]Choline incorporation dpm/mg protein (x10⁻³)
[³H]Choline labeling (h)
[³H]Choline labeling (h)

Figure 18

oleate-treated SRD 6 cells resulted in an initial elevation in [^{3}H]CDP-choline, but by 4 h levels had dropped back to those seen in CHO 7 cells (treated and untreated). In addition to enhanced synthesis of [^{3}H]PtdCho, oleate treatment also stimulated SM and GroPcho labeling in both cell lines. Surprisingly, [^{3}H]GroPcho levels were elevated to the greatest extent in oleate-treated SRD 6 cells.

We next examined the response of CT protein to oleate treatment to determine if increased PtdCho synthesis was due to increased enzyme translocation. Treatment of SRD 6 and CHO 7 cells with oleate (150 μM for 2 h) caused a slight decrease in immunoreactive CT in total cell lysates (Figure 19, panel A), and altered the isoform distribution such that the low molecular weight isoform of CT predominated in oleate-treated cells. Cytosolic immunoreactive CT almost disappeared completely after oleate treatment in both cell lines (Figure 19, panel B). This decrease in cytosolic CT was mirrored by increased membrane-bound CT (Figure 19, panel C). Membrane-bound CT from the oleate-treated SRD 6 and CHO 7 cells was predominately in the low molecular weight isoform compared to untreated cells.

Another potential *in vitro* lipid activator of CT is diacylglycerol (Cornell and Vance, 1987a). Diacylglycerol levels were measured in CHO 7 and SRD 6 cells grown with and without cholesterol using the diacylglycerol kinase assay (Table 8) to determine if this lipid was altered. There were no statistically significant differences between the amount of diacylglycerol in SRD 6 cells compared to CHO 7 cells, and cholesterol supplementation had no effect.

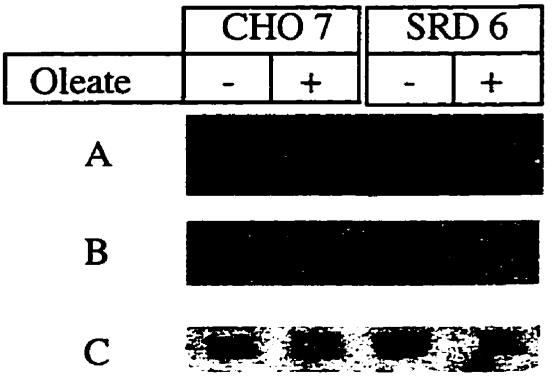

Figure 19. Effects of oleate on CT localization and mass in SRD 6 and CHO 7 cells. Cells were grown in medium B (without cholesterol) for 24 h, followed by treatment with (+) or without (-) 150 μM oleate complexed to bovine serum for 2 h. Total cell lysates (A), cytosol (B) and membranes (C) were prepared and 15 μg (10 μg for cell lysates) of protein from each fraction was subjected to 10% SDS-PAGE and immunoblotting as described in Materials and Methods. Similar results were seen in two other experiments.

Table 8. Diacylglycerol mass in CHO 7 and SRD 6 cells

Cell line +/- cholesterol	Diacylglycerol
	nmol / mg cell protein
CHO 7 -	1.36 ± 0.32
CHO 7 +	1.51 ± 0.46
SRD 6 -	1.65 ± 0.23
SRD 6 +	1.30 ± 0.05

Diacylglycerol mass from CHO 7 and SRD 6 cells grown with (+) or without (-) cholesterol (described in the legend for Figure 11) was assayed as described in Materials and Methods. Results are the average and range for two experiments.

C. Sphingomyelin synthesis and OSBP in SRD cells

1. Basal and 25-hydroxycholesterol stimulated SM synthesis in SRD cells.

Cholesterol and sphingomyelin have been shown to be coordinately regulated under numerous pathological and experimental conditions, and our laboratory has shown that SM synthesis is stimulated by 25-hydroxycholesterol (Ridgway, 1995b). 25-Hydroxycholesterol concentrations and treatment times required to stimulate SM synthesis were similar to that for inhibition of cholesterol synthesis and uptake, and activation of ACAT. The regulation of cholesterol and SM metabolism by oxysterols may proceed through the same mechanism. The SRD cells were used to investigate oxysterol-induced SM synthesis, and to determine if this response was dependent upon either cholesterol synthesis or cholesterol content. SM synthesis, and its stimulation by 25-hydroxycholesterol, was examined by [^{3}H]serine incorporation in CHO 7 and SRD cells grown in the presence or absence of cholesterol for 24 h (Table 9). Similar to wild-type CHO-K1 cells, SM synthesis in CHO 7 cells was stimulated by 25-hydroxycholesterol 1.5- to 2-fold regardless of cholesterol addition. In SRD 6 cells grown without cholesterol for 24 h, basal SM synthesis was reduced and the addition of 25-hydroxycholesterol further decreased [^{3}H]serine incorporation. Addition of cholesterol to SRD 6 cells had little effect on basal SM synthesis, which remained about one-half of activity in the CHO 7 cells, but oxysterol-induced SM synthesis was restored (2-fold). In SRD 2 cells, basal SM synthesis and the 25-hydroxycholesterol-induced increase in isotope

Table 9. [^{3}H]Serine incorporation into sphingolipids and phospholipids in CHO 7 and SRD cell lines.

	SM		GlcCer		Cer		PtdSer		PtdEtn	
	25-OH Cholesterol (2.5 μg/ml)									
	-	+	-	+	-	+	-	+	-	+
	dpm/nmol lipid phosphorus									
CHO 7-	126 ± 18	190 ± 30[a]	36 ± 6	35 ± 5	142 ± 20	111 ± 18	440 ± 123	419 ± 79	80±18	81 ± 6
CHO 7+	112 ± 34	203 ± 42[d]	29 ± 6	29 ± 4	112 ± 20	114 ± 9	378 ± 113	443 ± 153	70±19	85±15
SRD 6-	88 ± 24[3]	68 ± 27	91 ± 25[1]	59 ± 6	96 ± 17[4]	81 ± 15	347 ± 92	310 ± 137	67±16	74±34
SRD 6+	64 ± 18[2]	129 ± 29[e]	51 ± 20	51 ± 9	84 ± 20[3]	85 ± 4	351 ± 130	337 ± 144	64±25	63±30
SRD 2-	92 ± 7[4]	156 ± 25[c]	26 ± 9[4]	33 ± 12	158 ± 78	152 ± 97	262 ± 48[3]	270 ± 103	53 ± 5[3]	53 ± 7
SRD 4-	102 ± 10	236±11[b,3]	31 ± 9	45 ± 8	134 ± 12	133 ± 19	400 ± 67	505 ± 132	66 ± 5	81 ± 6[e]

Cells were cultured in medium B containing 42 μg cyclodextrin/ml (no cholesterol, -) or cholesterol (+, 2 μg/ml complexed to cyclodextrin, +) for 24 h. Cells were switched to serine-free medium B with (+) or without (-) 25-hydroxycholesterol ± cholesterol for 4 h. Cells were pulsed with 7.5 μCi/ml [^{3}H]serine for the last hour of this treatment, harvested and incorporation into lipids was assessed as described in Material and Methods. Results are the mean and standard deviation of 3-8 experiments. Significance was determined using a two-tailed *t*-test compared to untreated counterpart cells ([a]$P<0.001$, [b]$P<0.005$, [c]$P<0.01$, [d]$P<0.025$, [e]$P<0.05$), or compared to CHO 7- with or without 25-hydroxycholesterol ([1]$P<0.001$, [2]$P<0.005$, [3]$P<0.025$, [4]$P<0.05$).

incorporation were slightly decreased compared to CHO 7 cells. SRD 4 cells, when compared to CHO 7 cells did not have a significant difference in basal SM synthesis, but oxysterol stimulation was slightly higher (2.3-fold).

Compared to the other cell lines, SRD 6 cells grown in the absence of cholesterol had a 2.5-fold increase in [^{3}H]GlcCer production (Table 9). 25-Hydroxycholesterol had no effect on GlcCer synthesis except for the decrease in [^{3}H]GlcCer levels found in the cholesterol-deprived SRD 6 cells. Cholesterol supplementation of these cells partially corrected this elevation. The levels of [^{3}H]Cer in SRD 6 cells was 65-75% of control values. Isotope incorporation into Cer and GlcCer in SRD 4 was similar to CHO 7 cells, but in SRD 2 cells [^{3}H]Cer production was significantly decreased by 30%. PtdSer and PtdEtn labeling was similar in SRD 6, SRD 4 and CHO 7 cells, but significantly decreased by 40% in SRD 2 cells. Except for PtdEtn synthesis in SRD 4 cells, label incorporation into GlcCer, Cer, PtdSer and PtdEtn was not significantly altered with 25-hydroxycholesterol treatment.

Mevalonate is the product of HMG-CoA reductase, the rate-limiting enzyme of the cholesterol biosynthetic pathway. Addition of exogenous mevalonate to cells restores cholesterol production when this enzyme is inhibited (Brown et al., 1978). Thus, if cholesterol synthesis in the SRD 6 cells is blocked strictly by the absence of HMG-CoA reductase activity, and cholesterol synthesis is required for the regulation of SM production, then the addition of mevalonate may normalize SM synthesis. This hypothesis was tested by culturing SRD 6 cells in 1 mM mevalonate for 24 h prior to 25-hydroxycholesterol treatment

Figure 20. Activation of SM synthesis in CHO 7 and SRD 6 cells by 25-hydroxycholesterol in the presence of exogenous cholesterol or mevalonate. Cells were cultured in medium B containing 42 μg cyclodextrin/ml (no addition, open bars), cholesterol (2 μg/ml, black bars) or mevalonate (1 mM, light gray bars for 24 h. Cells then received serine-free medium B with the same additions containing 25-hydroxycholesterol (2.5 μg/ml) dissolved in ethanol or solvent alone for 4 h. During the last hour of oxysterol treatment cells were labeled with [^{3}H]serine (7.5 μCi/ml) and incorporation into SM (panel A), GlcCer (panel B) and Cer (panel C) was assessed. Results are the mean and standard deviation of four experiments, and shown as [^{3}H]serine incorporation relative to the non-oxysterol treated condition. Specific activities for SM, GlcCer and Cer were similar to the values shown in Table 9.

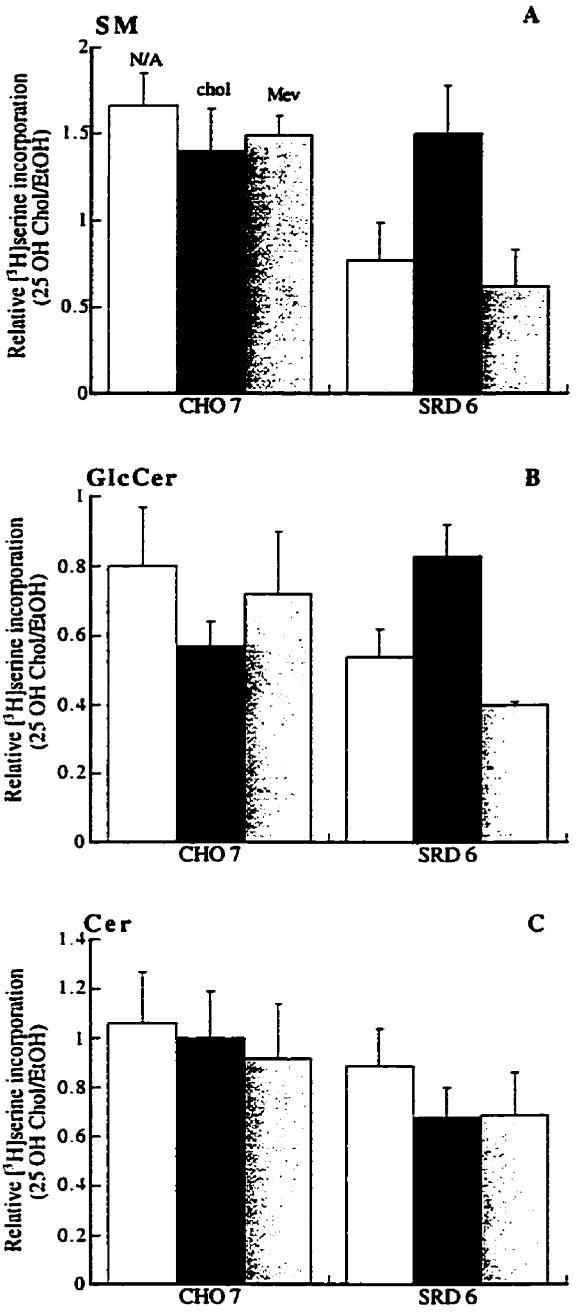
SM
A
N/A
chol
Mev
Relative [3H]serine incorporation (25 OH Chol/EtOH)
2
1.5
1
0.5
0
CHO 7
SRD 6
GlcCer
B
Relative [3H]serine incorporation (25 OH Chol/EtOH)
1
0.8
0.6
0.4
0.2
0
CHO 7
SRD 6
Cer
C
Relative [3H]serine incorporation (25 OH Chol/EtOH)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
CHO 7
SRD 6

Figure 20

(Figure 20). The results showed that mevalonate did not increase basal SM synthesis, nor did it restore the response to 25-hydroxycholesterol. Again, only cholesterol was effective in this regard. Mevalonate treatment did lower the amount of [^{3}H]GlcCer synthesized in the SRD 6 cells, but did not significantly alter labeling of Cer.

The differences in SM labeling observed in SRD 6 cells may be related to altered activities of the biosynthetic enzymes. The *in vitro* activities of serine palmitoyltransferase and SM synthase, the first and last steps of the SM pathway, were measured in the membrane fraction of CHO 7 and SRD 6 cells grown with and without cholesterol (Table 10). SRD 6 cells had reduced activity for both enzymes compared to CHO 7 cells regardless of cholesterol treatment, but these values were not statistically significant. The activities of these enzymes in both cell lines were unaffected by oxysterol treatment.

2. OSBP phosphorylation and localization in SRD 6 cells

From previous [^{3}H]serine incorporation studies it seemed likely that the site of 25-hydroxycholesterol action on SM synthesis was the conversion of ceramide to SM. This stimulation could involve either increased ceramide transport or increased conversion to SM at the Golgi apparatus (Ridgway, 1995b). Interestingly, 25-hydroxycholesterol is a ligand for OSBP, and promotes its translocation to the Golgi apparatus. Therefore, we tested whether OSBP expression and localization were affected by cholesterol auxotrophy, and whether these changes could be correlated with aberrant SM synthesis in SRD 6 cells. OSBP expression and phosphorylation were assessed by

Table 10. Serine palmitoyltransferase and SM synthase activity in CHO 7 and SRD 6 cells.

Cell line Chol/25-OH chol	Serine palmitoyltransferase	SM synthase
		pmol/min/mg protein
CHO 7 -/-	13.1 ± 6.0	28.6 ± 12.2
CHO 7 -/+	12.0 ± 5.9	30.0 ± 13.3
CHO 7 +/-	13.0 ± 6.1	33.1 ± 9.9
CHO 7 +/+	10.8 ± 4.1	26.6 ± 10.7
SRD 6 -/-	9.1 ± 4.1	17.8 ± 9.5
SRD 6 -/+	7.2 ± 7.2	17.1 ± 7.6
SRD 6 +/-	7.9 ± 3.8	11.7 ± 4.5
SRD 6 +/+	8.6 ± 3.3	15.6 ± 7.1

Cells were cultured in medium B with or without cholesterol (Chol, 2 μg/ml), prior to the addition of 25-hydroxycholesterol (25-OH chol. 2.5 μg/ml) for 4 h. Cells were harvested and the particulate membrane fraction was isolated and assayed for enzyme activities as described in Materials and Methods. Results are the mean and standard deviation of three experiments.

Figure 21. OSBP expression and phosphorylation in CHO 7 and SRD 6 cells. Cells were cultured in medium B containing either 42 μg cyclodextrin/ml (no cholesterol, -) or 2 μg cholesterol/ml complexed to cyclodextrin (+ cholesterol) for 24 h, followed by 25-hydroxycholesterol (2.5 μg/ml) or solvent control for an additional 4h. For immunoblot analysis, cells were harvested in 0.3% Triton X-100 buffer and equivalent amounts of protein (15 mg) were resolved by 6% SDS-PAGE, transferred to nitocellulose 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 weight isoform, respectively, by scanning densitometry and integration of peak size (average of 4 experiments). For [^{32}P]phosphate incorporation experiments (lower panel), cells were switched to phosphate-free medium for 1 h, and 100 μCi [^{32}P]phosphate/ml and oxysterol (2.5 g/ml) was added. After 4 h, cells were harvested, radiolabeled OSBP was immunoprecipitated from Triton X-100 extracts (35 μg protein) and resolved on 6% SDS-PAGE as described in Materials and Methods. The autoradiogram is the result of a 3 day exposure at -70°C.

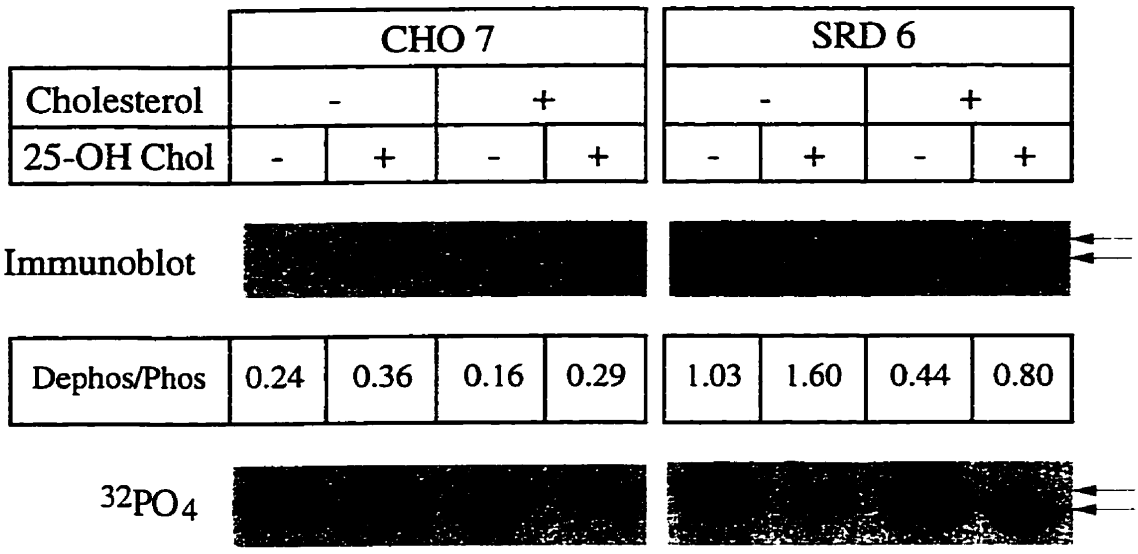
CHO 7
SRD 6
Cholesterol
-
+
-
+
25-OH Chol
-
+
-
+
-
+
-
+
Immunoblot
Dephos/Phos
0.24
0.36
0.16
0.29
1.03
1.60
0.44
0.80
32PO4

Figure 21

immunoblotting CHO 7 and SRD 6 cell extracts (Figure 21, panel A). OSBP migrates as a doublet on SDS-PAGE; the reduced mobility of the upper band of the doublet is the result of phosphorylation on at least 5 serine residues, while the lower band is hypophosphorylated and incorporates < 10% of total [^{32}P]phosphate (Ridgway et al., 1997). In CHO 7 cells, the majority (80%) of OSBP is in the hyperphosphorylated upper band. This is not affected by growth in cholesterol, and 25-hydroxycholesterol treatment for 4 h caused a slight shift in favor of the hypophosphorylated form. In contrast, OSBP from SRD 6 cells grown without cholesterol is distributed almost equally between the two forms. Total OSBP expression in SRD 6 cells was increased 2- to 4-fold compared to CHO 7 cells, and in cholesterol-depleted SRD 6 cells the amount of hyperphosphorylated OSBP was similar to CHO 7 cells. Growth of SRD 6 cells in cholesterol for 24 h restored the OSBP isoform distribution to 70% phosphorylated: 20% dephosphorylated. 25-Hydroxycholesterol treatment of SRD 6 cells, regardless of cholesterol addition, increased OSBP dephosphorylation to a greater extent than that observed in CHO 7 cells. [^{32}P]Phosphate labeling and immunoprecipitation confirmed the immunoblotting results (Figure 21, lower panel) [^{32}P]Phosphate incorporation into OSBP from SRD 6 cells grown without cholesterol and CHO 7 cells were similar. Cholesterol supplementation increased [^{32}P]phosphate incorporation into OSBP by 60-70% in SRD 6 cells. Consistent with immunoblot analysis, 25-hydroxycholesterol reduced [^{32}P]phosphate incorporation into OSBP in cholesterol depleted and cholesterol replete SRD 6 cells.

The changes in OSBP phosphorylation status in SRD 6 cells during a 24 h incubation in cholesterol-free medium was measured by shifts in distribution between the two OSBP isoforms (Figure 22). 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 weight form at 24 h. Initially, OSBP from SRD 6 cells grown in cholesterol (time 0) had a similar isoform distribution as CHO 7 cells and mass was elevated by approximately 2-fold. By 12 h in cholesterol-free medium there was a pronounced shift to the dephosphorylated isoform of OSBP that was maintained at 24 h.

In an effort to determine whether phosphorylation on particular serine residues in OSBP was cholesterol-sensitive, CHO 7 and SRD 6 cells were grown in the presence or absence of cholesterol for 24 h, labeled with [^{32}P]phosphate, digested with trypsin and the two-dimensional phosphopeptide map was examined (Figure 23). Similar to previous experiments (Ridgway et al., 1997) tryptic maps of [^{32}P]OSBP immunoprecipitated from CHO 7 cells showed the presence of 5 major phosphopeptides. OSBP from SRD 6 cells grown without cholesterol had a similar phosphopeptide map, and total isotope incorporation was increased compared to the CHO 7 phosphopeptide map (the exposure for the phosphopeptide map from SRD 6 cells was for 5 days versus 10 days for CHO 7 cells). The relative intensities of the [^{32}P]peptides were different, however. In SRD 6 cells grown without cholesterol, phosphopeptide 4 was decreased compared to CHO 7 cells and peptide 5 appeared to be absent. OSBP immunoprecipitated from cholesterol-supplemented SRD 6 cells

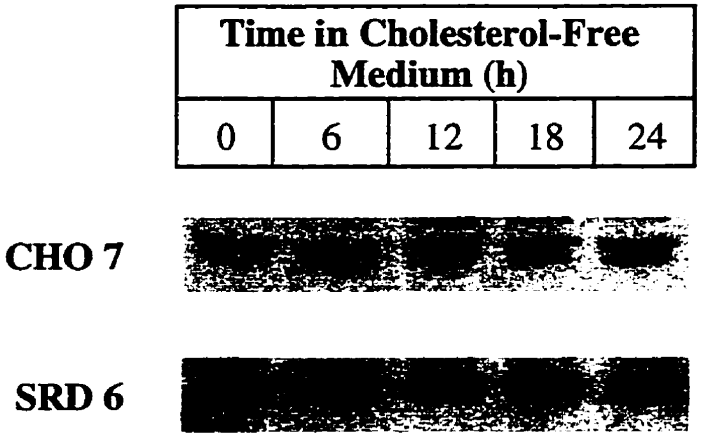

Figure 22. Time course for dephosphorylation of OSBP in cholesterol-free medium. CHO 7 and SRD 6 cells were grown in medium B with cholesterol (2 μg/ml complexed to cyclodextrin) for 3 days prior to the start of the experiment. At time 0, cells received cholesterol-free medium B and at the indicated times OSBP expression was quantitated in Triton X-100 cell extracts by immunoblotting as described in the legend to Figure 21.

Figure 23. Phosphopeptide maps of OSBP from CHO 7 and SRD 6 cells. Cells were cultured for 24 h in medium B with or without cholesterol. Cells were incubated with 400 μCi [^{32}P]phosphate for 4 h in phosphate-free medium, and OSBP was immunoprecipitated and digested with trypsin as described in Materials and Methods. The resultant phosphopeptides were separated on a cellulose TLC plate using electrophoresis in the first dimension, followed by thin-layer chromatography in the second dimension. The autoradiograms for the SRD 6 cells were the result of a 4 day exposure at -70°C with Kodak XAR film. For the CHO 7 autoradiogram the exposure time was 10 days.

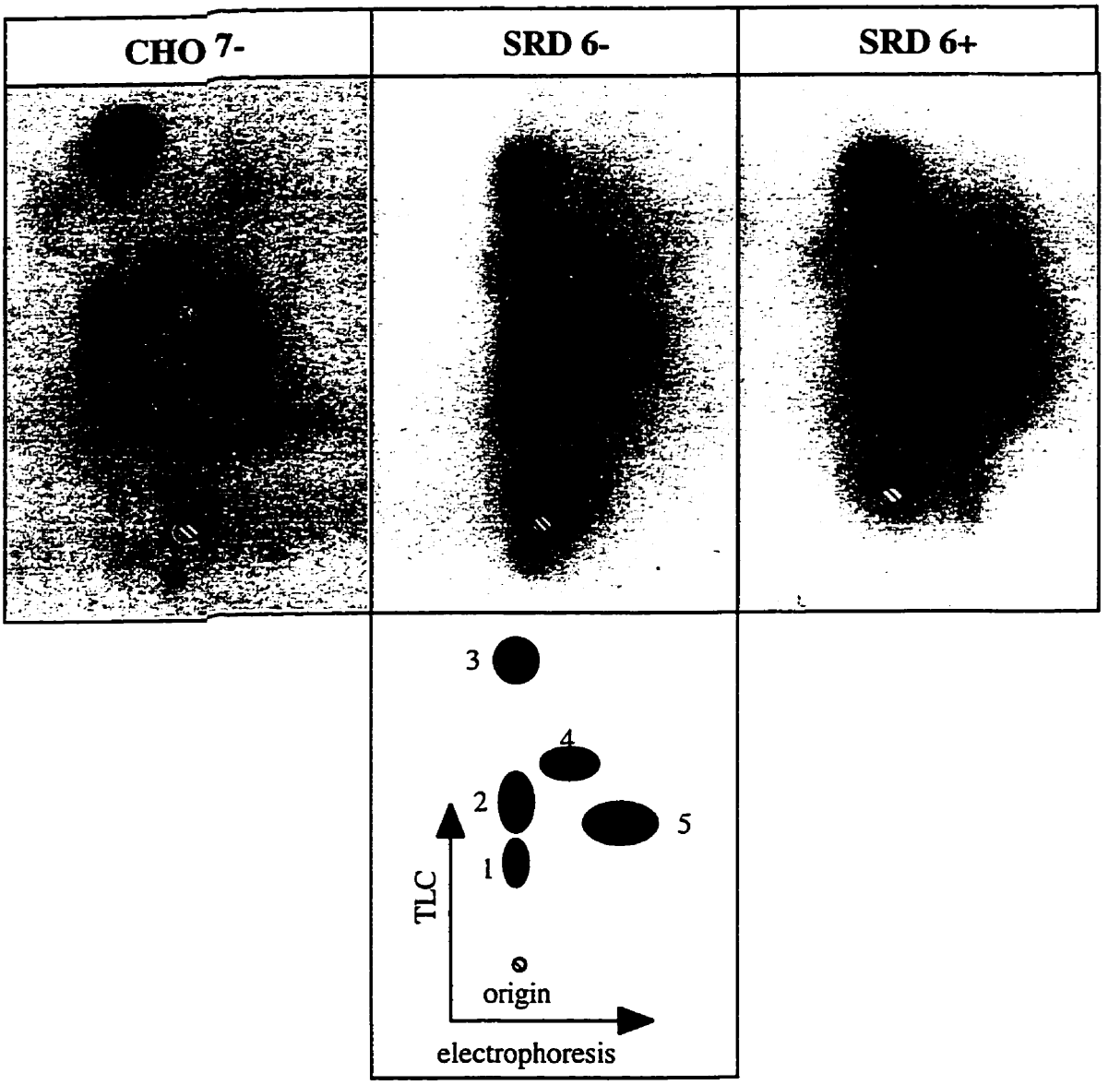

Figure 23

incorporated more label into all phosphopeptides, and relative intensities of phosphopeptides 4 and 5 were increased when compared to OSBP immunoprecipitated from SRD 6 cells grown without cholesterol.

OSBP undergoes translocation from a cytoplasmic/vesicular compartment to the Golgi apparatus in the presence of oxysterols. The effect of cholesterol depletion on translocation and localization of OSBP to these compartments was monitored in SRD 6 and CHO 7 cells by indirect immunofluorescence (Figure 24). As seen previously in studies with CHO-K1 cells that overexpress OSBP (Ridgway et al., 1992), untreated CHO 7 cells, regardless of cholesterol treatment, displayed a complex immunofluorescence pattern of small vesicles located around the nucleus and in the cytoplasm. 25-Hydroxycholesterol treatment for 4 h caused the immunofluorescence to shift to structures around or localized to one pole of the nucleus. This fluorescence pattern is similar to that for overexpressed OSBP and was shown to correspond to the Golgi apparatus (Ridgway et al., 1992). In contrast to CHO 7 cells, OSBP in SRD 6 cells grown in the absence of cholesterol displayed constitutive Golgi localization that was unaffected by 25-hydroxycholesterol. Cholesterol supplementation of SRD 6 cells for 24 h normalized OSBP localization, and OSBP translocated to the Golgi apparatus in response to 25-hydroxycholesterol.

The Golgi apparatus in SRD 6 and CHO 7 cells grown with or without cholesterol was similar as determined by staining with a Golgi-specific marker, FITC-labeled lentil lectin (Figure 25). In SRD 6 cells grown without cholesterol, the Golgi apparatus appeared more compact for both OSBP and lectin staining

Figure 24. Indirect immunofluorescence of OSBP in cholesterol-depleted CHO 7 and SRD 6 cells. Cells were cultured in medium B with either cholesterol (2 μg/ml, complexed with cyclodextrin, +) or cyclodextrin alone (42 μg/ml, -) for 24 h. Cells were then treated with 25-hydroxycholesterol (2.5μg/ml) or solvent (ethanol) for 4 h. Cells were processed for immunofluorescence using affinity purified ab104 as described in Materials and Methods.

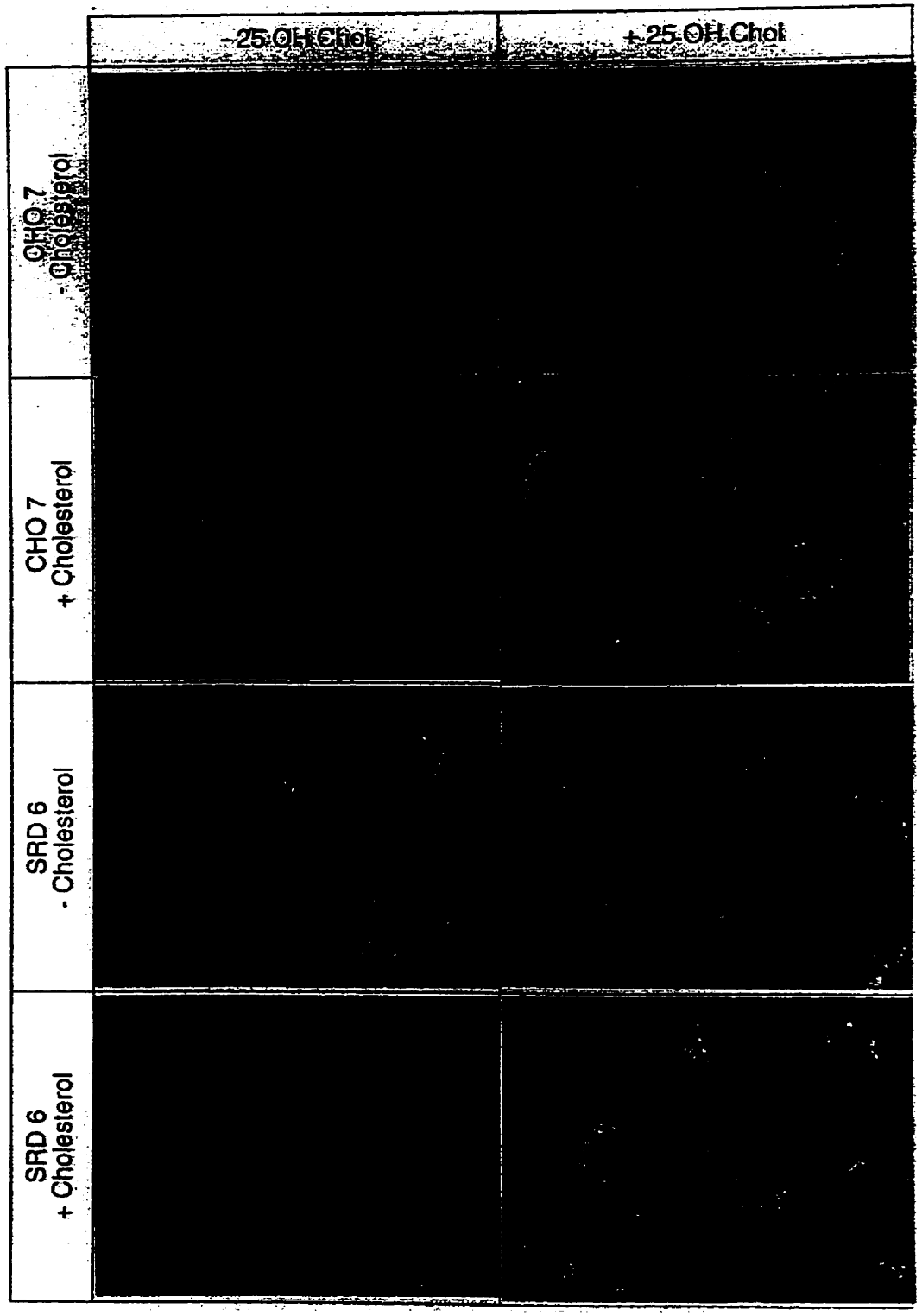
-25-OH Chol
+25-OH Chol
CHO 7
- Cholesterol
CHO 7
+ Cholesterol
SRD 6
- Cholesterol
SRD 6
+ Cholesterol

Figure 24

Figure 25. Indirect immunofluorescence of the Golgi apparatus in CHO 7 and SRD 6 cells. Cells were cultured in medium B with either cholesterol (2 μg/ml, complexed with cyclodextrin, +) or cyclodextrin alone (42 μg/ml, -) for 24 h. Cells were processed for immunofluorescence using FITC-labeled lentil lectin as described in Materials and Methods.

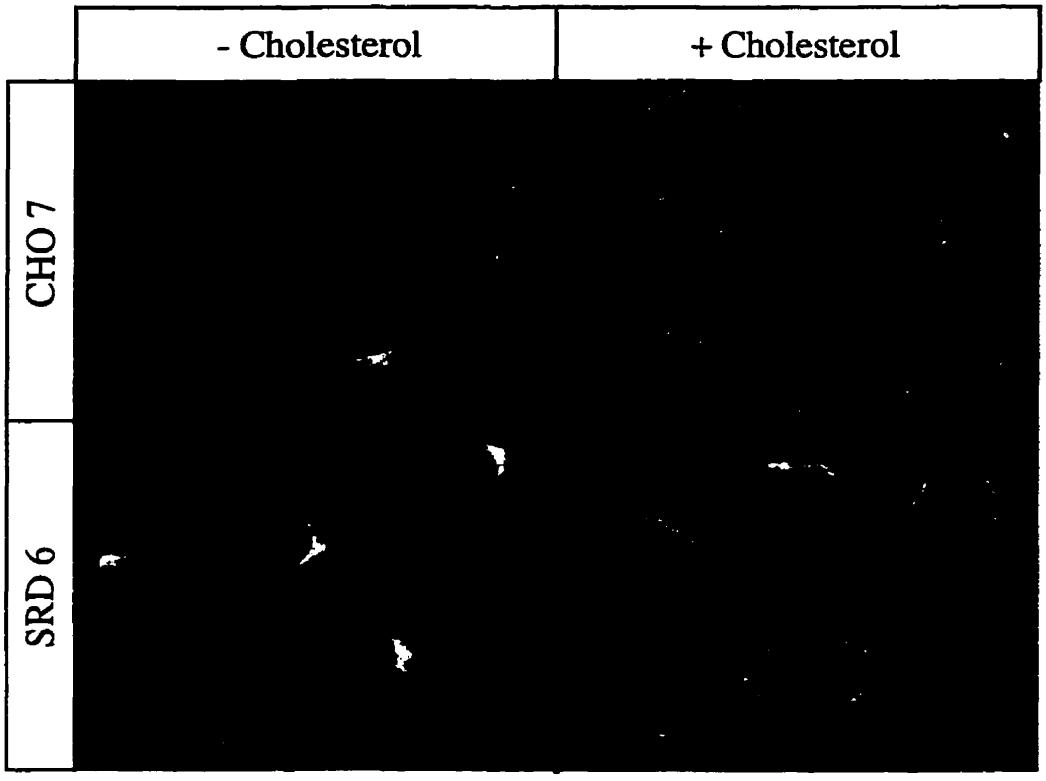

Figure 25

(Figure 24 and 24). This difference, however, was slight indicating that the SRD 6 mutation did not greatly affect the morphology of this organelle.

D. Effect of lovastatin on SM metabolism and OSBP regulation.

1. Effects of lovastatin on SM synthesis in CHO-K1 cells

Since cholesterol supplementation restores 25-hydroxycholesterol activation of SM synthesis in SRD 6 cells, this effect is most likely due to low cholesterol content. In an effort to prove this and mimic the SRD 6 phenotype, wild-type CHO-K1 cells were treated with drugs that alter cholesterol synthesis and trafficking. Lovastatin, a competitive HMG-CoA reductase inhibitor, blocks cholesterol synthesis and, when used in combination with lipoprotein-free medium, decreases cellular cholesterol levels (Grundy, 1988; Brown et al., 1978). The treatment regime used in the present study was 0-50 μM lovastatin for a 12 h period prior to 25-hydroxycholesterol addition for 4 h. SM synthesis was then quantitated by [^{3}H]serine incorporation (Figure 26, panel A). Lovastatin caused increased basal SM synthesis at low concentrations (0.1 μM), while higher concentrations (>10 μM) decreased basal synthesis dramatically. As previously documented, 25-hydroxycholesterol addition to CHO-K1 stimulated SM synthesis 2-fold (Ridgway, 1995b). Lovastatin decreased the response to 25-hydroxycholesterol, and with 50 μM the amount of [^{3}H]SM synthesized in 25-hydroxycholesterol treated cells was similar to non-oxysterol treated values. Lovastatin concentrations of 1 μM or more, inhibited incorporation of [^{14}C]acetate into cholesterol by >90% (Figure 26, panel B). The lovastatin concentrations used in these experiments had no effect on cell

Figure 26. SM synthesis in lovastatin-treated CHO-K1 cells. CHO-K1 cells were cultured in medium A for 3 days, followed by medium B (5% LPDS) for 12 hours with the indicated concentrations of lovastatin. Cells were then incubated for 4 h in the presence of 25-OH cholesterol (2.5 μg/ml, ■), or solvent control (❑) and SM synthesis (panel A) was assessed as described in the Table 9. Results are the mean and standard deviation of three separate experiments. Cholesterol synthesis (panel B) was quantitated by the incorporation of [^{14}C]acetate (100 μM with 2.5 μCi/ml for 2 h) as described in Materials and Methods. Results are the average of duplicate dishes from a representative experiment. Total cellular protein as a measure of cell growth (panel C) was determined. Results are the average of duplicate samples from a representative experiment.

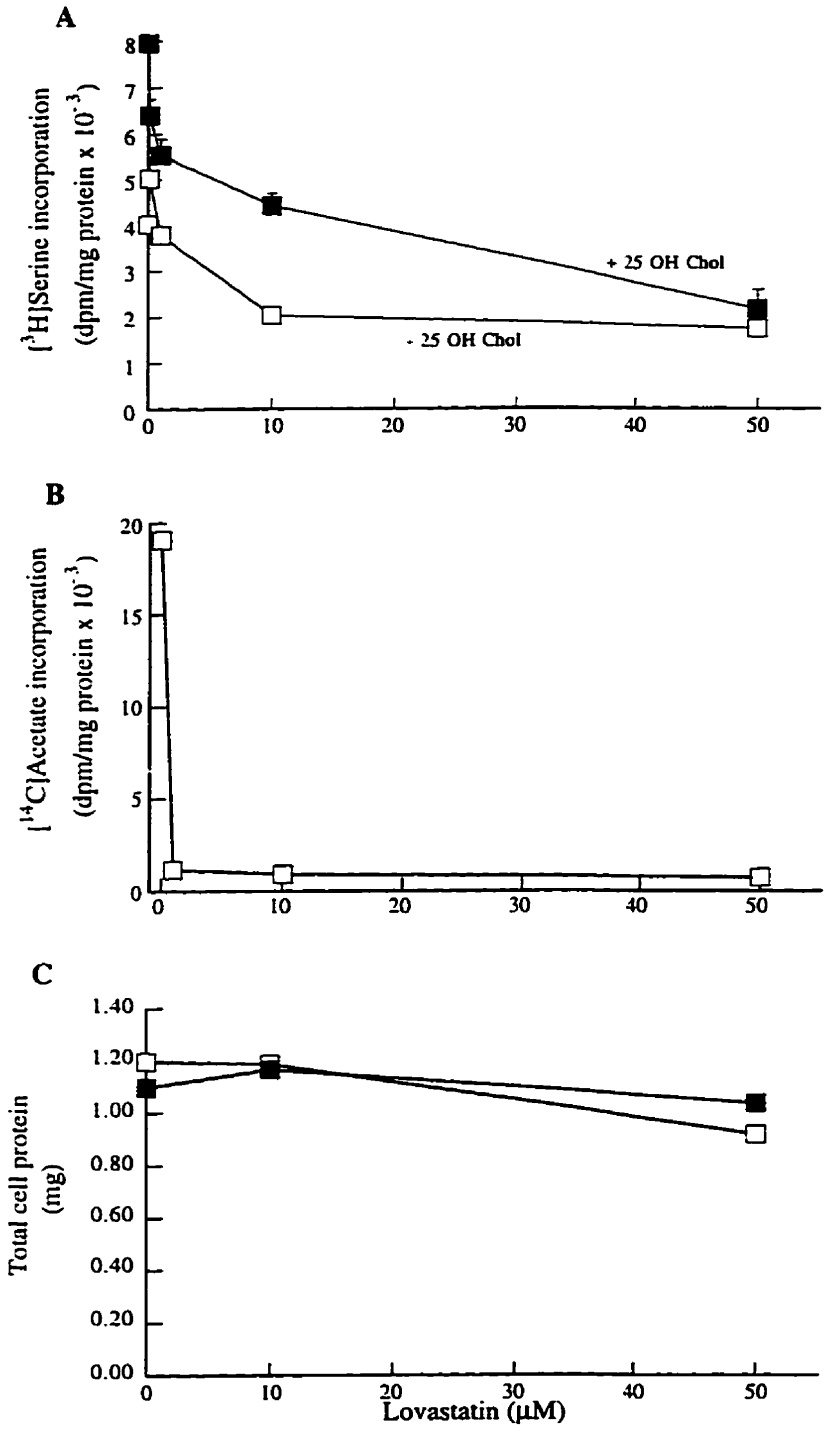
A
[3H]Serine incorporation (dpm/mg protein x 10-3)
+ 25 OH Chol
- 25 OH Chol
B
[14C]Acetate incorporation (dpm/mg protein x 10-3)
C
Total cell protein (mg)
Lovastatin (μM)

Figure 26

Table 11. [^{3}H]Serine incorporation into phospholipids and ceramide in lovastatin-treated CHO-K1 cells.

Lovastatin (μM)	PtdSer		PtdEtn		Cer	
	25-OH Cholesterol (2.5 μg/ml)					
	-	+	-	+	-	+
	(dpm/ mg protein x 10^{-3})					
0	13 ± 3	14 ± 1	3.1 ± 0.4	3.8 ± 0.8	6.1 ± 0.8	6.6 ± 1.1
0.1	14 ± 2	13 ± 3	3.7 ± 1.0	3.9 ±0.4	5.4 ± 1.2	5.5 ± 0.9
1	12 ± 2	12 ± 2	3.3 ± 0.8	3.4 ±0.4	5.3 ± 1.5	5.8 ± 1.5
10	11 ± 3	12 ± 3	3.0 ± 0.6	3.1 ± 0.9	3.9 ± 0.7[a]	4.4 ± 2.2
50	12 ± 2	11 ± 1	2.9 ± 1.0	2.4 ± 0.6	3.4 ± 1.4[b]	3.1 ± 1.4[b]

CHO-K1 cells were cultured in medium B (5% LPDS) for 12 h with various concentrations of lovastatin, treated with (+) or without (-, solvent control) 25-hydroxycholesterol (2.5 μg/ml) for 4 h, and 7.5 μCi [^{3}H]serine for the last 1 h. [^{3}H]Seine incorporation into PtdSer, PtdEtn and Cer was quantitated as described in Materials and Methods. Results are the mean and standard deviation of three experiments. Significance was determined using a two-tailed *t*-test compared to non-lovastatin treated controls ([a]$P<0.025$, [b]$P<0.05$).

growth, as determined by the cellular protein content (Figure 26, panel C). [^{3}H]Serine incorporation into PtdSer and PtdEtn was not altered by 25-hydroxycholesterol, or lovastatin treatment (Table 11). Isotope incorporation into Cer was inhibited by lovastatin to a maximum of 50% of control values, but did not vary with 25-hydroxycholesterol treatment.

The alterations in SM synthesis caused by lovastatin were probably due to decreased cholesterol content, but it is also possible that active synthesis of cholesterol played a role. To differentiate these possible mechanisms, lovastatin experiments were repeated in cells cultured with either whole serum, or LPDS with 1 mM mevalonate (Figure 27). Culturing cells with whole serum and lovastatin restored the 2-fold stimulation in SM synthesis by 25-hydroxycholesterol, but the basal rate of SM synthesis was still decreased by 40%. Treatment with mevalonate in combination with lovastatin had no effect on basal SM synthesis compared to non-lovastatin treated controls, and the addition of oxysterol also did not alter [^{3}H]SM synthesis. CHO-K1 cells cultured in whole serum had a greater activation of SM synthesis by 25-hydroxycholesterol compared to cells cultured in LPDS, and in mevalonate-treated cells. [^{3}H]Cer synthesis was still decreased by lovastatin treatment of cells cultured in whole serum, but mevalonate normalized Cer synthesis in non-oxysterol treated cells. The presence of 25-hydroxycholesterol in combination with lovastatin stimulated Cer synthesis in cells cultured in whole serum, but decreased synthesis in mevalonate treated cells.

Figure 27. SM synthesis in CHO-K1 cells treated with lovastatin under different growth conditions. CHO-K1 cells were cultured in medium A (whole serum), medium B (LPDS,) or medium B containing mevalonate (1 mM mevalonate) for 12 h with or without lovastatin. 25-Hydroxycholesterol (2.5 μg/ml, or solvent control) was then added and cells were incubated for an additional 4 h, with [^{3}H]serine (7.5 μCi/ml) present for the last hour. [^{3}H]Serine incorporation into SM (panel A) and Cer (panel B) was quantitated as described in Materials and Methods. Results are the mean and standard deviation of three experiments and expressed as [^{3}H]serine incorporation relative to the non-oxysterol, non-lovastatin treated condition (- lovastatin, + 25-OH chol, open bars; + lovastatin,- 25-OH chol, black bars; + both, gray bars).

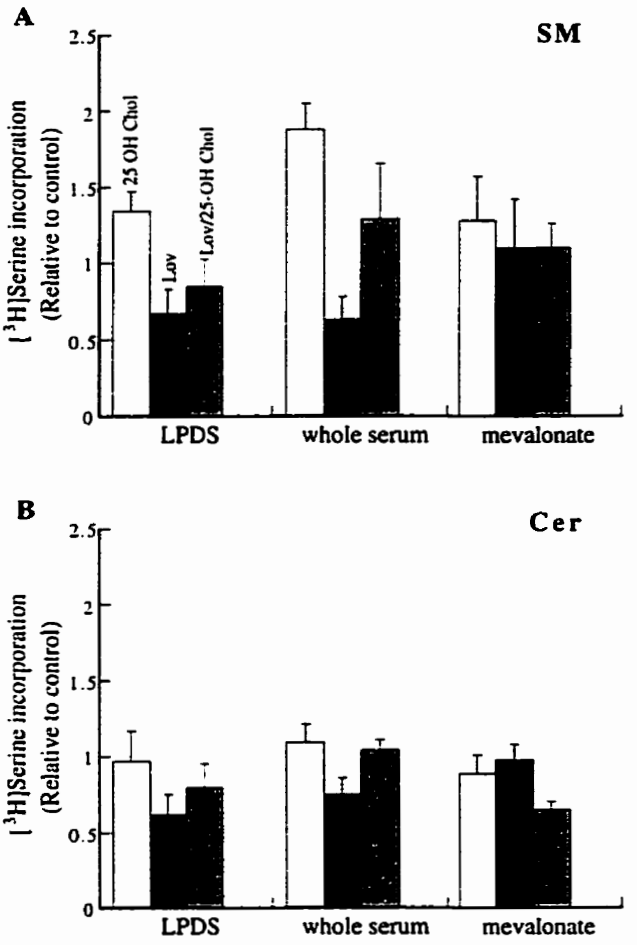
A
SM
[³H]Serine incorporation (Relative to control)
2.5
2
1.5
1
0.5
0
25 OH Chol
Lov
Lov/25-OH Chol
LPDS
whole serum
mevalonate
B
Cer
[³H]Serine incorporation (Relative to control)
2.5
2
1.5
1
0.5
0
LPDS
whole serum
mevalonate

Figure 27

2. OSBP phosphorylation and expression in CHO-K1 cells treated with lovastatin

Lovastatin-treated CHO-K1 cells were examined for effects on OSBP expression. Triton X-100 cellular extracts from cells cultured in LPDS and treated for 12 h with increasing concentrations of lovastatin, prior to 25-hydroxycholesterol treatment, were immunoblotted for OSBP (Figure 28, Figure 29A). These immunoblots showed that >1 μM lovastatin increased the amount of dephosphorylated OSBP (Figure 28). This effect appeared to be independent of 25-hydroxycholesterol treatment.

To determine if cholesterol synthesis or decreased cholesterol content was responsible for lovastatin-induced dephosphorylation of OSBP, immunoblotting experiments were repeated with the addition of mevalonate and whole serum in the medium. The addition of mevalonate, but not whole serum, inhibited the shift in OSBP phosphorylation status caused by lovastatin (Figure 29A). As observed for OSBP from CHO 7 cells, 25-hydroxycholesterol treatment caused a slight shift toward a more dephosphorylated distribution in CHO-K1 cells under all treatment conditions. Immunoprecipitaton of [^{32}P]phosphate-labeled OSBP showed that lovastatin treatment, in combination with mevalonate and LPDS, had little effect on OSBP phosphorylation (Figure 29B). However, lovastatin treatment reduced [^{32}P]phosphate incorporation into OSBP in cells cultured in whole serum.

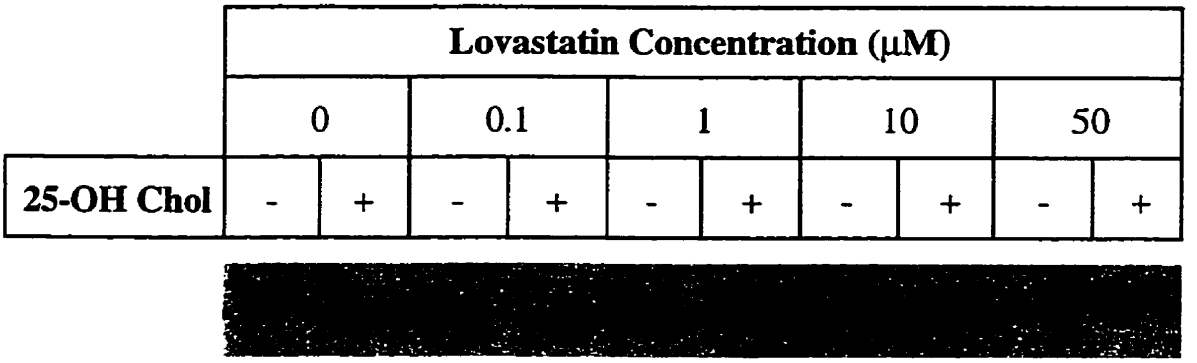

Figure 28. Effects of lovastatin treatment on OSBP phosphorylation in CHO-K1 cells. CHO-K1 cells were grown in medium B containing various concentrations of lovastatin for 12 h prior to the treatment for 4 h with 25-hydroxycholesterol (+, 2.5 μg/ml) or solvent alone (-). OSBP expression was assessed in Triton X-100 cell extracts by immunoblotting as described in Materials and Methods using 15 μg of protein.

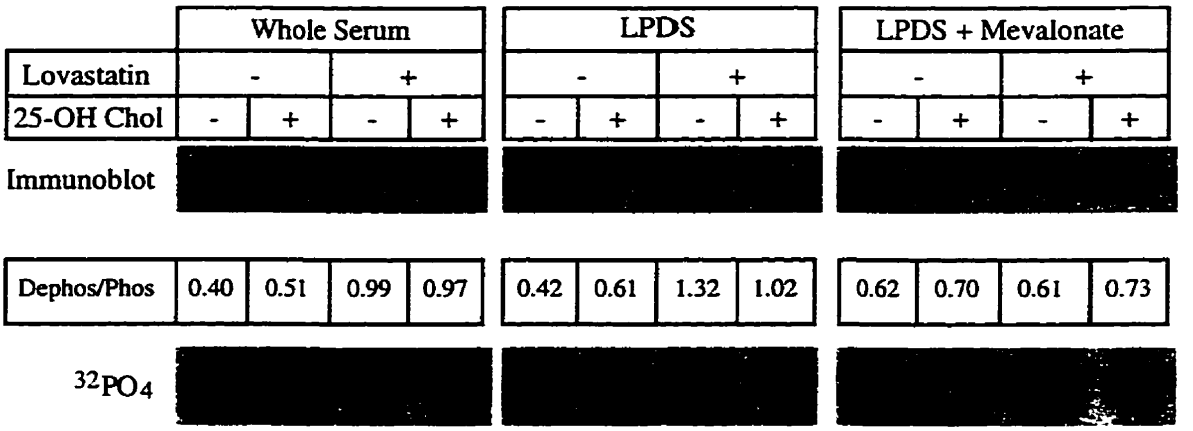

Figure 29. OSBP expression and phosphorylation in Lovastatin-treated CHO-K1 grown in various sterol conditions. Cells were cultured in either medium A (containing whole serum, 5% FCS), medium B (containing 5% LPDS) or medium B containing 1 mM mevalonate (LPDS + mevalonate) for 12 h with (+) or without (-) 50 μM lovastatin. 25-Hydroxycholesterol (2.5 μg/ml or solvent control) was then added and cells were incubated for an additional 4 h. OSBP expression was assessed by immunoblotting 15 μg of protein as described in Materials and Methods. The ratio of dephosphorylated to phosphorylated OSBP (Dephos/Phos) was determined from the amount of the low and high molecular weight isoforms respectively (average of 2 experiments). For [^{32}P]phosphate incorporation (lower panel) 35 μg of protein was immunoprecipitated as described in the legend to Figure 21. The autoradiogram is the result of a 3 day exposure at -70°C with Kodak BioMax film.

E. Effects of U18666A on SM synthesis and OSBP.

1. SM synthesis in CHO-K1 cells treated with U18666A

The hydrophobic amine U18666A inhibits cholesterol synthesis and intracellular cholesterol transport, and caused accumulation of LDL-derived cholesterol in the lysosome (Phillips and Avigan, 1963; Liscum and Faust, 1989). To investigate if normal cholesterol trafficking was required for regulation of basal and 25-hydroxycholesterol-stimulated SM synthesis, increasing concentrations of U18666A were used to pre-treat (15 min) CHO-K1 cells cultured in either whole serum or LPDS, before 25-hydroxycholesterol addition. Basal [^{3}H]serine incorporation into SM in CHO-K1 cells cultured in whole serum (Figure 30, panel A) was stimulated (4-fold) by increasing U18666A concentrations. The oxysterol-induced activation of SM synthesis in these cells decreased slightly in the presence of the drug. Due to these combined responses, [^{3}H]SM synthesis in both oxysterol and non-oxysterol treated cells were similar at 5 μg U18666A/ml. In cells cultured with LPDS (Figure 30, panel B), U18666A (concentrations of 2 μg/ml or more) also inhibited 25-hydroxycholesterol activation of SM synthesis. However, basal [^{3}H]SM synthesis was unaltered. Basal ACAT activity in cells cultured in whole serum or LPDS was maximally inhibited at a concentrations of 0.5 μg U18666A/ml (Figure 30, panel C). As previously documented (Liscum and Faust, 1989), treatment with U18666A does not appreciably affect 25-hydroxycholesterol activation of ACAT.

Figure 30. Effects of U18666A treatment on SM synthesis in CHO-K1 cells. CHO-K1 cells were grown in medium A (whole serum, ■,□) or in medium B (LPDS, ●,○) for 24 h. Cells were then incubated in either serine-free of serine-containing medium A or B with varying amounts of U18666A for 15 min (or with a DMSO control), prior to the addition of 25-hydroxycholesterol (2.5 μg/ml, closed symbols or solvent, open symbols) for 4 h. SM synthesis (panels A and B) was assessed by [^{3}H]serine incorporation (7.5 μCi/ml for 1 h) as described in Materials and Methods. Results are the mean and standard deviation of three experiments. ACAT activity (panel C) was measured by the incorporation of [^{3}H]oleate into cholesterol ester as described in Materials and Methods. Results are the average and range of two experiments.

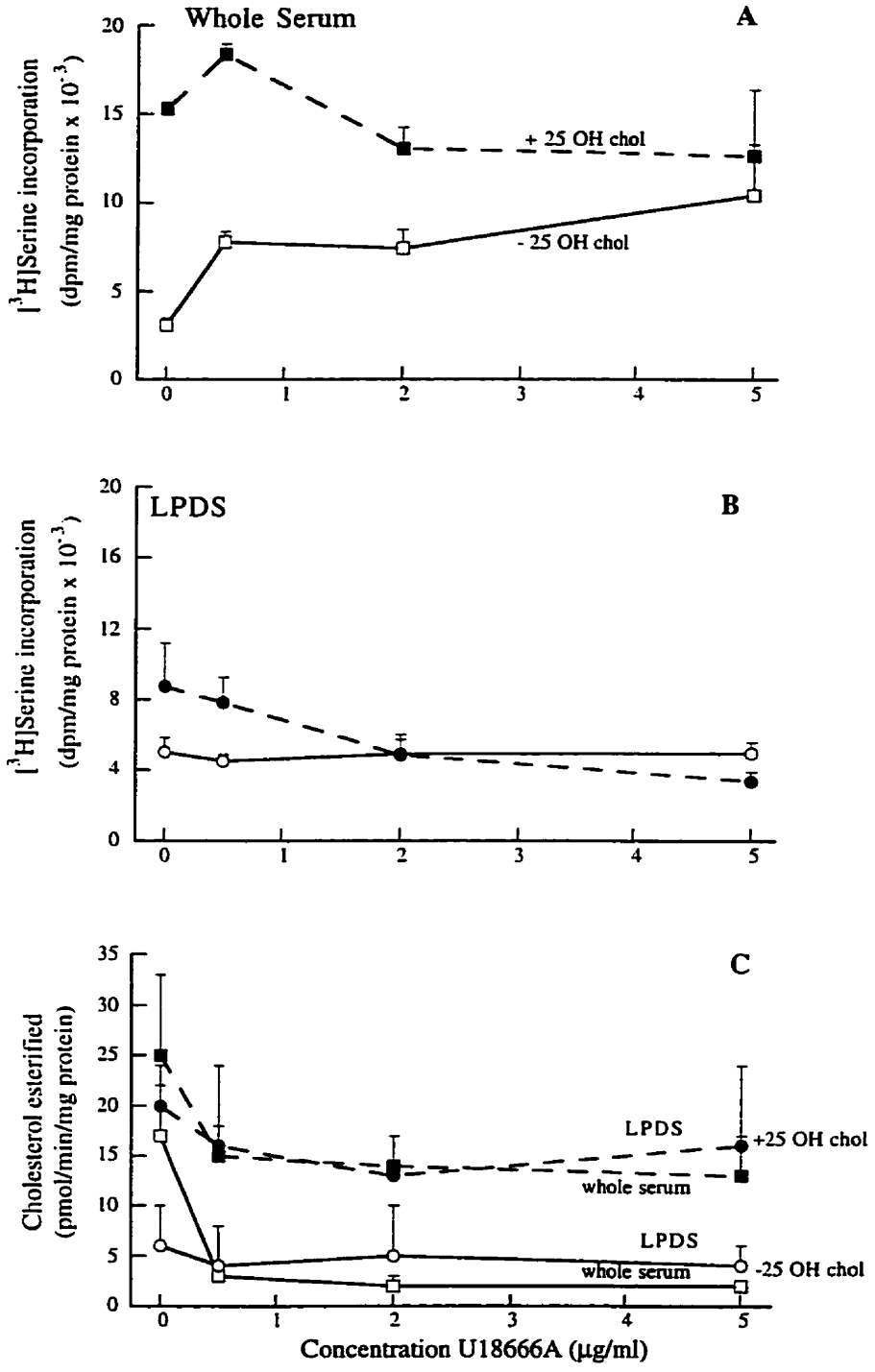
Whole Serum
A
[3H]Serine incorporation (dpm/mg protein x 10-3)
+ 25 OH chol
- 25 OH chol
LPDS
B
C
Cholesterol esterified (pmol/min/mg protein)
LPDS
+25 OH chol
whole serum
LPDS
whole serum
-25 OH chol
Concentration U18666A (µg/ml)

Figure 30

Table 12. Effect of U18666A on [^{3}H]serine labeling of lipids in CHO-K1 cells.

U18666A (μM)	PtdSer		PtdEtn		GlcCer		Cer	
	25-OH Cholesterol (2.5 μg/ml)							
	-	+	-	+	-	+	-	+
	dpm/mg protein x 10^{-3}							
Whole Serum								
0	56 ± 8	51.9 ± 0.8	7.9 ± 0.6	7.6 ±4.0	1.3 ± 0.4	2.8 ± 0.9	4.6 ± 1.6	6.9 ± 3.6
0.5	62 ± 9	63 ± 26	6.8 ± 3.0	10.2 ± 3.1	3.1 ± 0.4	3.6 ± 1.4	6.0 ±3.0	7.3 ± 3.8
2	54 ± 14	50 ± 11	7.9 ± 2.0	7.5 ± 1.6	3.6 ± 0.5	3.4 ± 0.6	5.7 ± 3.1	5.5 ± 0.5
5	59 ± 13	53 ± 10	8.7 ± 0.4	8.9 ± 2.7	4.2 ± 2.8	3.2 ± 2.2	5.3 ± 2.2	5.2 ± 2.5
LPDS								
0	41 ± 17	40 ± 14	5.6 ± 0.7	6.0 ± 0.3	2.1 ± 1.2	1.9 ± 1.8	7.1 ± 1.9	6.5 ± 3.2
0.5	43 ± 2	45 ± 4	5.9 ± 0.3	6.5 ± 2.0	2.2 ± 1.7	2.0 ± 1.0	5.4 ± 2.7	6.2 ± 0.2
2	53 ± 18	54 ± 17	6.5 ± 0.9	7.0 ± 0.5	2.2 ± 1.3	1.6 ± 0.8	4.0 ± 0.5	4.1 ± 1.1
5	61 ± 20	46 ± 8	7.8 ± 1.0	6.2 ±0.5	2.1 ± 0.9	1.1 ± 0.4	4.2 ± 0.5	3.5 ± 1.0

CHO-K1 cells were treated and labeled as described in the legend for Figure 30. [^{3}H]Serine incorporation was quantitated as described in Materials and Methods. Results are the average and range from two experiments.

U18666A (μg/ml)	0		0.5		2		5	
25-OH Chol	-	+	-	+	-	+	-	+
Whole Serum								
Protein	1.0	0.8	1.4	1.6	2.1	1.8	2.1	1.7
Dephos/Phos	0.17	0.19	0.30	0.31	0.36	0.34	0.49	0.36
LPDS								
Protein	1.0	1.1	0.8	0.6	1.2	0.8	1.2	1.0
Dephos/Phos	0.19	0.16	0.48	0.48	0.46	0.58	0.65	0.68

Figure 31. OSBP expression and phosphorylation in CHO-K1 cells treated with U18666A. CHO-K1 cells were grown in medium A (whole serum, with LDL) or medium B (LPDS, without LDL) for 24 h. Cells were then treated with various amount of U18666A for 15 min prior to the addition of 25-hydroxycholesterol (2.5 μg/ml or solvent) for 4 h. Immunoblotting of OSBP was performed as described in the legend to Figure 21. The relative mass changes (Protein, compared to untreated controls) and the ratio of dephosphorylated to phosphorylated OSBP (Dephos/Phos) was determined by scanning. Similar results were seen in one other experiment.

As seen in other [^{3}H]serine incorporation experiments, labeling of PtdEtn and PtdSer was not affected by either oxysterol or U18666A treatment (Table 12). In non-oxysterol treated cells cultured in whole serum, [^{3}H]GlcCer levels increased approximately 3-fold with U18666A treatment. Addition of 25-hydroxycholesterol seemed to inhibit this increase. In whole serum, [^{3}H]Cer levels were similar under all treatment conditions. In cells cultured in LPDS, GlcCer synthesis was unaffected by U18666A treatment, but Cer synthesis was decreased by 50-60%, regardless of oxysterol treatment.

2. OSBP expression and phosphorylation in U18666A-treated CHO-K1 cells

Treatment of CHO-K1 cells with U18666A affected OSBP phosphorylation and expression in a cholesterol-dependent manner (Figure 31). Cells cultured in whole serum showed a dose-dependent 2-fold increase in OSBP mass after treatment with U18666A for 4.25 h. The OSBP distribution ratio between the hyperphosphorylated and dephosphorylated isoforms was increased by 2-fold, and 25-hydroxycholesterol treatment did not have an effect. Cells that were grown in LPDS and treated with U18666A had similar amounts of immunoreactive OSBP compared to controls, but the presence of U18666A caused a 3-fold increase in the dephosphorylated to phosphorylated OSBP ratio. Again, 25-hydroxycholesterol did not affect OSBP phosphorylation under these conditions.

V. Discussion

A greater understanding of how cholesterol and phospholipid synthesis are coordinately regulated is important for basic knowledge of how cholesterol and SM metabolic pathways interact in the cell, and to our understanding of diseases caused by aberrant cholesterol or phospholipid homeostasis. For example, it is well established that PtdCho, SM and cholesterol accumulate in atherosclerotic lesions and foam cells (Shiratori et al., 1994; Smith and Cantab 1960; Smith 1974; McCandless and Zilversmit, 1956). Also, in the Niemann-Pick disorders cholesterol, sphingolipids and phospholipids accumulate irrespective of whether the defect is in cholesterol (NP type II) or sphingomyelin (NP type I) metabolism (Spence and Callahan, 1989). However, little is known about the biochemical mechanisms underlying these changes in lipids and sterol levels, or the effect of altered *de novo* cholesterol synthesis on the metabolism of phospholipids. Using the SRD mutants, which have altered cholesterol metabolism, and drugs which alter cholesterol synthesis and trafficking we have demonstrated a series of relationships between PtdCho, SM, cholesterol and fatty acid metabolism.

A. Cholesterol metabolism in SRD cells.

Studies with CHO cell mutants have already provided important information about the mechanism and control of cholesterol regulation (Evans and Metherall, 1993; Sakai, et al., 1996; Metherall et al., 1989; Leonard and Sinensky, 1988) and trafficking (Dahl et al, 1992), and phospholipid

biosynthesis (Zoeller and Raetz, 1992). The SRD cells used for this study have well characterized defects in cholesterol regulation and responses to 25-hydroxycholesterol and other regulatory sterols. The SRD 6 defect results from incomplete proteolytic cleavage of SREBP-1 and 2 to the mature nuclear-localized transcription factors (Sakai et al., 1996). SRD 6 cells have low mRNA levels for HMG-CoA reductase, HMG-CoA synthase, LDL receptor and farnesyl diphosphate synthase, but post-transcriptional regulation of ACAT and HMG-CoA reductase is normal (Evans and Metherall, 1993). Sterol synthesis in SRD 6 cells is 1% of that in the parental cell line. As a result, these cells are cholesterol auxotrophs. Due to the lack of active SREBP transcription factors, fatty acid synthesis in SRD 6 cells is also compromised.

SRD 2 cells do not down-regulate transcription of the sterol-regulated genes in response to 25-hydroxycholesterol. The mutation in the SRD 2 is the result of recombination of SREBP-2 with an unidentified gene, which produces a truncated SREBP-2 protein (Yang et al., 1995). This mutant protein contains the full transcriptional activator domain of the SREBP, but lacks the transmembrane domain and is free to enter the nucleus without a requirement for proteolysis. Thus cholesterol and fatty acid synthesis are increased 3- and 2.5-fold, respectively. The basal rate of cholesterol esterification in the SRD 2 cells was increased, but remained responsive to 25-hydroxycholesterol. The increase in basal ACAT activity is likely the result of increased cholesterol synthesis within these cells. Due to a functioning ACAT protein, unesterified cholesterol showed modest accumulation in cells grown in LPDS (Jackson et al., 1996).

SRD 4 cells are also 25-hydroxycholesterol resistant. Like SRD 2 cells, oxysterol inhibition of sterol-regulated gene transcription is absent. This results from a point mutation in the sterol-cleavage activator protein (SCAP) (Nohturfft et al., 1996). SCAP is a transmembrane protein, which forms a complex with the full-length SREBPs and initiates the first proteolytic step, causing the transcription factor domain of the SREBPs to be released from the membrane (Sakai et al., 1997). The SRD 4 mutation in SCAP produces a dominantly active protein that is not responsive to sterols (Nohturfft et al., 1996). SRD 4 cells also lack ACAT activity due to a point mutation in one allele of the ACAT gene (Cao et al., 1996). The other ACAT allele is in a region of a chromosome that is heavily methylated (Cao et al., 1996) and thereby transcriptionally silenced, leaving the cells functionally hemizygous (Holliday, 1991).

B. Coordinate regulation of cholesterol, phosphatidylcholine and fatty acid metabolism in SRD cells.

Using the cholesterol auxotrophic SRD 6 cells, we demonstrated a relationship between PtdCho, cholesterol and fatty acid metabolism by which a defect in processing of SREBP results in decreased production of PtdCho via the CDP-choline pathway. When depleted of cholesterol, SRD 6 cells have reduced PtdCho synthesis and a resultant 8% decrease in PtdCho mass. Inclusion of cholesterol in culture medium partially restored the reduction in PtdCho mass, but not the decreased PtdCho synthesis.

In sterol-depleted SRD 6 cells, the mass of the other phospholipids was either increased or unaltered, and [^{3}H]serine incorporation into PtdEtn and

PtdSer was also not affected. Thus, the decrease in PtdCho synthesis and mass in sterol-depleted SRD 6 cells was specific for this lipid. Protein synthesis in SRD 6 cells, cultured in the absence and presence of cholesterol was similar to that of controls, and decreased DNA synthesis in these cells was unaffected by cholesterol treatment.

The total phospholipid:protein ratio in SRD 6 cells depleted of cholesterol was similar to parental cells and cholesterol-supplemented SRD 6 cells. The total phospholipid:cholesterol ratio is increased 1.7-fold in sterol-depleted SRD 6 cells compared to control. Decreased PtdCho mass in the sterol-depleted SRD 6 cells partially compensates for decreased cholesterol content. Even with decreased PtdCho mass, this ratio was still slightly increased; the PtdCho:cholesterol ratio was 3.3 for sterol-depleted SRD 6 cells, 2.6 for CHO 7 cells grown without cholesterol and 1.7 for SRD 6 cells grown with cholesterol. Sterol-depleted SRD 6 cells, even with decreased PtdCho synthesis and mass, were unable to maintain a constant PtdCho:cholesterol ratio. Therefore, in SRD 6 cells the lipid composition of membranes is altered, since these cells cannot fully compensate for the large cholesterol decrease.

The decrease in PtdCho synthesis in SRD 6 cells was due to inhibition of CDP-choline synthesis as demonstrated by 1) an accumulation of [^{3}H]Pcho, 2) delayed synthesis of [^{3}H]PtdCho observed in [^{3}H]choline pulse-chase experiments, and 3) an increase in Pcho mass. These results were consistent with reduced activity of CTP:phosphocholine cytidylyltransferase (CT) *in vivo*. While CT activity appeared to be reduced in intact SRD 6 cells, it was normal or

elevated when assayed *in vitro* in total membranes or cytosol, respectively. This could indicate that *in vivo,* the membrane-bound form is relatively inactive, or the soluble enzyme is restricted in its capacity to translocate to membranes in SRD 6 cells. It is also possible that during cellular fractionation we removed a factor or modified CT, which could explain the lack of correlation between the *in vitro* and *in vivo* results. Another explanation for these differences is that CT in SRD 6 cells translocated to a different cellular compartment, and therefore would not have proper access to its substrates and activators.

There are several explanations for reduced PtdCho synthesis in SRD 6 cells and its lack of response to exogenous cholesterol. First, a product of the isoprenoid biosynthetic pathway, not the end product cholesterol, may be required for full restoration of PtdCho synthesis. In these experiments cells were incubated with cholesterol and 1 mM mevalonate, or minus sterols and mevalonate. Since the rate of transcription initiation for HMG-CoA reductase and HMG-CoA synthase in the SRD 6 cells are decreased and non-existent, respectively, these cells cannot synthesize isoprenoids without the addition of mevalonate or HMG-CoA. The capacity of SRD 6 cells to utilize exogenous mevalonate for isoprenoid was not efficient since mRNA for farnesyl diphosphate synthase, another key enzyme of the isoprenoid pathway, was almost nonexistent. Therefore, it is likely that mevalonate addition cannot fully restore isoprenoid synthesis. If a product of the isoprenoid pathway is required for normal PtdCho synthesis, the likely candidates are farnesyl and geranylgeranyl groups. These isoprenoids are added post-translationally to

numerous proteins involved in signal transduction and growth regulation (Casey, 1992). The enzymes in the CDP-choline pathway are not modified with isoprenyl groups, but a regulatory protein that alters this pathway could be. Second, active cholesterol synthesis within the ER could be is a prerequisite for PtdCho synthesis. The lipid composition of the ER and activity of enzymes in this organelle may be dependent on *de novo* cholesterol synthesis and transport. How this would regulate either PtdCho synthesis or CT activity is unclear. Third, aberrant regulation of PtdCho synthesis could be due to altered cholesterol distribution within the cell. Cholesterol in SRD 6 cells is obtained via diffusion, thus these cells may have abnormal cholesterol localization compared to normal. The exogenous cholesterol may localize to different organelles or regions of the PM, and not enter the cellular cholesterol trafficking pool. Thus, even though these cells are replete of cholesterol, the cells are not sensing this and PtdCho synthesis remains down-regulated.

The most likely explanation for the results in SRD 6 cells, is that PtdCho synthesis is inhibited by lack of a fatty acid-derived activator. Sterols have been shown to suppress transcription of fatty acid synthase (Bennett et al., 1995), and acetyl CoA carboxylase (Lopez et al., 1996), two key enzymes in fatty acid biosynthesis. This inhibition is modulated via SREBP transcription factors. In agreement with this we found that synthesis of fatty acids in the SRD 6 cells was decreased 70% compared to controls. SREBPs are not involved in CT expression since mRNA levels were not altered in cells lines with constitutive or absent activation of these transcription factors. Fatty acids and diacylglycerol

are know to activate PtdCho synthesis by promoting the translocation of CT to membranes and activating the enzyme (Vance, 1989; Pelech et al., 1983a; Sleight and Kent 1980). Thus, when levels of fatty acid or a fatty acid derivative are reduced, a mechanism exists for down-regulating CDP-choline production by maintaining CT in a inactive form. Membranes from SRD 6 cells appeared to contain similar levels of CT, but cytosolic CT was increased 2-fold and PtdCho synthesis was decreased. Thus *in vivo* CT activity did not strictly correlate with absolute levels of membrane-associated CT. SRD 6 cells contained similar levels of diacylglycerol compared to control cells, but it is possible that diacylglycerol contents within specific organelles or membrane regions, may be altered. However, treatment of these cells with oleate for as little as 2 h normalized PtdCho synthesis and stimulated CT translocation to membranes. Oleate treatment may also result in remodeling of lipid composition. Thus, either oleate or an oleate derived product directly activated CT most likely because of increased CT translocation or activation was more efficient due to altered fatty levels.

Alterations in the kinetic properties of SRD 6 CT may account for the altered PtdCho synthesis observed within these cells. Kinetic analysis of membrane-bound CT showed that the K_m for both CTP and Pcho in SRD 6 cells was similar to controls, and only the V_{max} for Pcho was decreased by 30%. This change in V_{max} may partially account for decreased CT activity, but likely is not the complete explanation for a 50% decrease in PtdCho synthesis, or the massive elevation of [^{3}H]Pcho in SRD 6 cells.

Phosphorylation appears to be another important mechanism for regulation of CT activity and PtdCho synthesis. Although CT is potentially phosphorylated on 16 serines, studies indicated that mutation of these serines to alanines or conversion to a constitutive phosphorylation phenotype (glutamic acid) does not completely override membrane/cytosol localization or seriously affect enzyme activity (Wang and Kent, 1995b). Instead, phosphorylation may have subtle effects on the lipid associated activation of the enzyme. Dephosphorylation of membrane CT was proposed to account for stimulation of PtdCho synthesis in cholesterol-loaded macrophages (Shiratori et al., 1995). In this model, enzyme mass was similar in membranes and cytosol from control and cholesterol-loaded macrophages, but membrane-bound CT was substantially dephosphorylated relative to controls resulting in increased catalytic activity (Shiratori et al., 1995). This study also showed that CHO-K1 cells do not respond to cholesterol-loading in this manner. Consistent with this, we also did not observe differences in phosphorylation status between the CHO-derived SRD 6 and CHO 7 cells that would account for decreased PtdCho synthesis. Differences in CT activity found in SRD 6 cell fractions relative to CHO 7 cells could be accounted for on the basis of increased enzyme mass. However, CT isolated from SRD 6 cytosol displayed lower specific radioactivity after [^{32}P]phosphate incorporation compared to controls. This may indicate that dephosphorylation of SRD 6 CT is favored in an attempt to overcome the block in enzyme activation.

Oleate treatment of SRD 6 cells also stimulated [^{3}H]GroPcho production. This indicates that increased PtdCho synthesis is accompanied by PtdCho catabolism, even when cells have decreased PtdCho mass. Thus, a possible explanation for decreased PtdCho mass in cholesterol-depleted SRD 6 cells is the inability of these cells to coordinate PtdCho synthesis and catabolism.

In the 25-hydroxycholesterol-resistant SRD 2 and SRD 4 cells we demonstrated that PtdCho metabolism is also affected by constitutive activation of SREBPs. Both SRD 2 and SRD 4 cells displayed elevated [^{3}H]GroPcho levels, which indicates elevated PtdCho catabolism. Both cells have constitutive transcription of sterol-regulated genes, but SRD 4 cells synthesized more [^{3}H]GroPcho. Thus, altered regulation of PtdCho metabolism in SRD 4 cells may be due to elevated unesterified cholesterol in the ER due to the ACAT mutation or the dominant mutation in SCAP possibly the increased SREBP-1 activation. Increased fatty acid synthesis may also activate PtdCho catabolism, since SRD 2 cells synthesize twice as much fatty acid compared to control and had slightly elevated PtdCho degradation. This increase in PtdCho catabolism may be in response to fatty acid-induced PtdCho synthesis.

SRD 4 cells also display alterations is PtdCho synthesis. First, CT activity appeared to be elevated due to increased [^{3}H]choline incorporation into CDP-choline and flux through the CDP-choline pathway appeared to be increased since total [^{3}H]choline incorporation was elevated as well. However, PtdCho mass and [^{3}H]PtdCho levels in these cells were similar to those in CHO 7 cells. Regulation of CT also seemed to be slightly altered in these cells, with an

approximate 20% decrease in cytosolic activity and a corresponding increase in membrane activity (these differences were not statistically significant). Also the CT isoform distribution in SRD 4 cells indicated increased CT dephosphorylation compared to controls and SRD 2 cells. This alteration in CT phosphorylation was similar to that seen in membranes from other cells displaying increased PtdCho synthesis (Shiratori et al., 1994). Thus in SRD 4 cells, there appears to be increased in PtdCho synthesis, but it is uncertain if increased catabolism is resulting from or stimulating increased synthesis.

Our results on CT activity and PtdCho synthesis in SRD 6 cells offer some interesting correlations to findings in cholesterol-loaded macrophages (Shiratori et al., 1994) and livers of cholesterol-fed rats (Lim et al, 1983) regarding cholesterol availability and PtdCho synthesis. In these systems PtdCho synthesis was increased, and the proportion of CT activity in membranes was also elevated. The opposite was true in SRD 6 cells; the proportion of CT activity and mass was increased in the inactive cytoplasmic pool, and PtdCho mass and synthesis were reduced. The large pool of cytosolic CT in SRD 6 cells is suggestive of an inability to translocate to membranes. As previously mentioned this could be related to lack of an appropriate lipid activator or abnormal composition of intracellular membranes.

A model for co-regulation of PtdCho synthesis and cholesterol metabolism is shown in Figure 32. Decreased PtdCho synthesis in the cholesterol auxotrophs is not a direct result of decreased cholesterol, but a down-stream effect of decreased SREBP activation. The SREBPs regulate both cholesterol and fatty

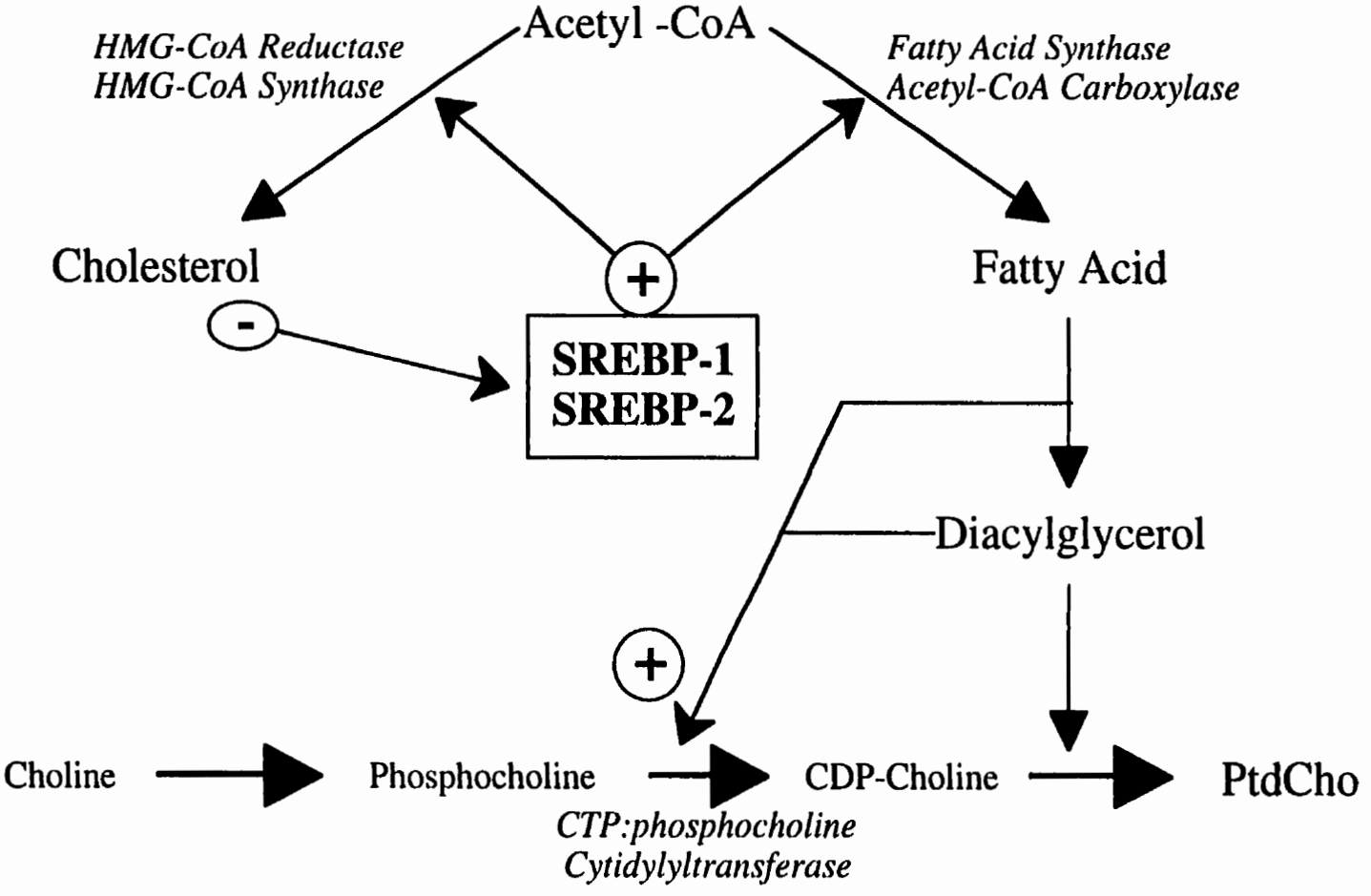

Figure 32. Proposed Mechanism for PtdCho, cholesterol and fatty acid co-regulation

acid synthesis, and by decreasing fatty acid content of the cell, a specific activating signal required for normal CT activity is removed. The regulation of PtdCho metabolism in cholesterol auxotrophic cells demonstrates the interconnected regulation of phospholipid, cholesterol and fatty acid metabolism.

In summary, cholesterol auxotrophy due to lack of transcriptional induction of sterol-regulated genes results in reduced PtdCho synthesis and mass in mutant SRD 6 cells. A combination of reduced ACAT activity and constitutive transcription of the same genes results in increased PtdCho degradation in SRD 4 and SRD 2 cells.

C. Coordinate regulation of cholesterol and SM metabolism and role of OSBP

Lovastatin and U18666A, drugs that alter cholesterol synthesis and intracellular trafficking, and the SRD cell lines were used to investigate the relationships between cholesterol regulation, SM metabolism and the oxysterol receptor, OSBP. Using these cell models, we demonstrated a direct relationship between expression, phosphorylation and localization of OSBP, and the ability of 25-hydroxycholesterol to activate SM synthesis. Furthermore, phosphorylation and intracellular localization of OSBP to the Golgi apparatus was affected by cholesterol levels. This implicates OSBP as a potential regulator of both the sterol and SM pathways. These results also showed a direct correlation between cholesterol availability and transport, and regulation of basal and 25-hydroxycholesterol-stimulated SM synthesis.

The effects observed in this study related to changes in both basal SM synthesis and its stimulation by 25-hydroxycholesterol. In SRD 6 cells grown either in the presence or absence of cholesterol, basal SM synthesis was reduced 30-40% compared to controls. This could result from similar reductions (not statistically significant) in the *in vitro* activities for SPT and SM synthase, two key enzymes in the SM biosynthetic pathway. Since SM mass in SRD 6 cells was normal, and appeared unaffected by cholesterol, changes in sphingolipid catabolism may compensate for this synthetic defect. Basal SM synthesis could not be stimulated by exogenous cholesterol, thus it would appear that active *de novo* cholesterol synthesis is required to correct this reduction. As discussed in Section B, SRD 6 cells also had reduced PtdCho synthesis. Thus, reduced [^{3}H]serine incorporation into SM may also be the result of restricted PtdCho availability.

We used lovastatin, an HMG-CoA reductase inhibitor, in an attempt to reproduce the SRD 6 phenotype in wild-type CHO-K1 cells. As expected, lovastatin treatment blocked cholesterol synthesis and at the same time altered SM synthesis. Inhibition of HMG CoA reductase by lovastatin decreased basal SM synthesis by 40-50% and this was only restored by the addition of mevalonate. Thus, regulation of basal SM synthesis is dependent on active cholesterol synthesis.

Treatment of CHO-K1 cells with U18666A, which inhibits both cholesterol synthesis and transport was used to determine if active cholesterol transport was required for regulation of SM synthesis. These experiments showed that

regulation of basal SM synthesis is not simply dependent upon cholesterol synthesis. When this drug was used in combination with lipoprotein-containing serum, basal SM synthesis was increased. When it was used in lipoprotein-free serum, however, basal synthesis was unaffected. It is possible U18666A did not have large effects on cholesterol synthesis due to the short treatment times, and that the effects seen on basal SM synthesis were strictly due to alterations in cholesterol transport and the accumulation of unesterified cholesterol within the lysosomes (Liscum and Faust, 1989; Liscum and Collins, 1991; Lange et al., 1991). Interestingly, U18666A treatment of fibroblasts mimics the NP-type II phenotype, where lysosomal cholesterol accumulation promotes SM accumulation (Liscum and Faust, 1989; Liscum and Collins, 1991). The SM accumulation in U18666A-treated and NP-type II cells may result from stimulation of basal synthesis.

U18666A did not affect basal SM synthesis in cells cultured in LPDS. Thus, it could be the lack of an isoprenoid that is regulating basal SM synthesis in SRD 6 and lovastatin-treated cells. U18666A inhibits squalene cyclase (Phillips and Avigan, 1963), thus the non-sterol isoprenoids are synthesized. In lovastatin treated cells and SRD 6 cells, HMG-CoA reductase activity is decreased, thus any product down-stream from this enzyme may be the regulating factor. Since mevalonate treatment of the SRD 6 cells did not restore basal SM synthesis, this compound could not be the regulator. As mentioned above, mevalonate in these cells will not be efficiently converted to isoprenoids

since farnesyl diphosphate synthase was poorly transcribed. Thus, the regulator of SM basal synthesis may be a non-sterol isoprenoid product.

Distinct from the effect of the SRD 6 mutation on basal SM synthesis was the lack of response to 25-hydroxycholesterol in cholesterol-depleted SRD 6 cells, and its restoration by exogenous cholesterol. Given that 25-hydroxycholesterol stimulated ceramide conversion to SM (Ridgway, 1995b), cholesterol would appear to be required for some component of this process. Further support for the concept that 25-hydroxycholesterol stimulation of SM synthesis is cholesterol-dependent comes from the finding that CHO 7 cells grown in lipoprotein-deficient medium displayed a blunted response compared to either SRD 4 cells or CHO-K1 cells grown in the presence of lipoproteins. In both lovastatin and U18666A experiments, CHO-K1 controls grown in lipoprotein-deficient medium also showed decreased oxysterol-induced stimulation of SM synthesis compared to controls grown in whole serum.

Lovastatin treatment of CHO-K1 cells mimics the SRD 6 phenotype with respect to 25-hydroxycholesterol stimulation of SM synthesis. Inhibition of cholesterol synthesis by lovastatin abolishes the 25-hydroxycholesterol stimulation of SM synthesis, but only in the absence of lipoproteins. Since both mevalonate and whole serum restores the 25-hydroxycholesterol stimulation of SM synthesis, total cholesterol content of the cell must be the regulating factor. If cholesterol synthesis was required, whole serum would not have restored this response. This dependency on cholesterol content for 25-hydroxycholesterol stimulation also requires active cholesterol transport. U18666A treatment

decreased 25-hydroxycholesterol activation of SM synthesis in the presence and absence of lipoproteins. Therefore it is not the total amount of cholesterol present that is regulating, but its localization within the cell.

To summarize, regulation of SM synthesis is dependent upon cholesterol in two ways. First, basal synthesis is dependent upon both cholesterol synthesis and cholesterol transport. Second, the stimulation of SM synthesis by 25-hydroxycholesterol requires the presence of cholesterol and active cholesterol transport. In both cases, the localization of cholesterol within the cell is as important as the concentration.

We could not identify the enzymes of SM synthesis affected by cholesterol or 25-hydroxycholesterol. As in CHO-K1 cells (Ridgway, 1995b), SM synthase and SPT were unaffected by 25-hydroxycholesterol or growth in cholesterol in both CHO 7 and SRD 6 cells. SM synthase activity is unlikely to be directly affected by cholesterol since reconstitution in PtdCho vesicles with up to 15 mol% cholesterol did not alter activity (Hanada et al., 1991).

The synthesis of GlcCer also appears to be regulated by cholesterol. In SRD 6 cells depleted of cholesterol, [^{3}H]GlcCer levels were elevated and this was partially corrected by exogenous cholesterol. In U18666A-treated cells cultured in whole serum, [^{3}H]GlcCer levels were also elevated. This effect on GlcCer synthesis was normalized by the addition of oxysterols, which was interesting since in all other cells and drug treatments, 25-hydroxycholesterol did not have an effect on either GlcCer or Cer synthesis. U18666A treatment of cells cultured in lipoprotein-deficient serum did not effect GlcCer levels, but Cer synthesis was

decreased. Lovastatin treatment also exhibited decreased Cer synthesis. These results suggest that ceramide metabolism at the Golgi is sterol-regulated and conversion to SM and GlcCer requires active cholesterol transport.

In an effort to identify the mechanism for 25-hydroxycholesterol stimulation of SM synthesis and the role of cholesterol, OSBP was studied in SRD 6 cells and CHO-K1 cells treated with lovastatin and U18666A. The site of action of 25-hydroxycholesterol on SM synthesis appears to be the conversion of ceramide to SM, a reaction that takes place in the *cis/medial* Golgi elements (van Echten and Sandhoff, 1993; Bell et al., 1993b; Futerman et al., 1990). This conclusion was reached in CHO-K1 cells, based on enhanced conversion of [^{3}H]sphinganine-derived ceramide to SM (Ridgway, 1995b). OSBP may play a pivotal role in the regulation of SM synthesis by oxysterols since it binds 25-hydroxycholesterol with high affinity (10 nM) (Taylor et al., 1984) and translocates from a cytosolic/vesicular compartment to the Golgi apparatus (Ridgway et al., 1992). Further evidence for a role of OSBP in SM regulation came from CHO-K1 cells overexpressing OSBP. These cells displayed a 3- to 4-fold increase in SM synthesis (measured by [^{3}H]serine incorporation) in response to 25-hydroxycholesterol relative to mock transfected cells (Storey et al., In preparation).

OSBP was partially dephosphorylated and constitutively Golgi-localized under the conditions of cholesterol depletion in SRD 6 cells. In accordance with the hypothesis that OSBP plays a role in the regulation of SM synthesis, these same conditions resulted in the absence of oxysterol-induced SM synthesis. It is

likely that the abnormal OSBP localization was related to its altered phosphorylation that occurs in cholesterol depleted cells. While the absolute level of phosphorylated OSBP is similar in CHO 7 and cholesterol-depleted SRD 6 cells, phosphopeptide maps revealed that phosphorylation of two peptides were specifically reduced in SRD 6 cells. This phosphorylation was restored by growth in cholesterol. Total phosphorylation of all sites in OSBP was increased in SRD 6 cells grown with cholesterol, and SRD 6 cells had increased OSBP mass compared to CHO 7 cells regardless of cholesterol treatment.

Lovastatin treatment of CHO-K1 cells also caused dephosphorylation of OSBP, as seen by immunoblotting, but this was not always correlated with [^{32}P]phosphorus incorporation. This lack of correlation between isoform distribution and OSBP phosphate incorporation could be due to an imperfect relationship between dephosphorylation of serines on OSBP and the shift in isoform distribution seen by SDS-PAGE. Also, OSBP was dephosphorylated to a greater extent in cholesterol-depleted SRD 6 cells compared to lovastatin-treated cells, so perhaps the immunoprecipitation experiments were not sensitive enough to display these subtle changes in OSBP phosphorylation. Lovastatin-induced dephosphorylation of OSBP was inhibited by the addition of mevalonate to the medium. However, addition of lipoprotein-containing serum to the culture conditions did not normalize OSBP phosphorylation. For SM synthesis, the additions had the opposite effect; lipoprotein-containing serum restored 25-hydroxycholesterol stimulation, whereas in mevalonate-treated

cells, SM synthesis was resistant to 25-hydroxycholesterol treatment. Lovastatin treatment had no effect on OSBP mass. This lack of correlation between dephosphorylation and 25-hydroxycholesterol resistant SM synthesis in lovastatin treated cells could be due to a lesser degree of OSBP dephosphorylation compared to sterol-depleted SRD 6 cells.

In U18666A-treated cells cultured in lipoprotein-deficient serum, OSBP was dephosphorylated and SM synthesis was not stimulated by 25-hydroxycholesterol. In whole serum, phosphorylation of OSBP was reduced, OSBP mass increased and SM synthesis was not stimulated by 25-hydroxycholesterol. Collectively, these results indicate that low cholesterol content, inhibition of cholesterol synthesis, and the inhibition of cholesterol trafficking decreases OSBP phosphorylation. Most importantly, a decrease in OSBP phosphorylation correlated with the inability of 25-hydroxycholesterol to stimulate SM synthesis.

Increased OSBP mass seen in SRD 6 cells cannot explain the lack of 25-hydroxycholesterol stimulation, since OSBP overexpressing cells displayed an elevated and more rapid increase in SM synthesis in response to 25-hydroxycholesterol (Storey et al., in preparation). In addition to altered SM regulation, these same overexpressing cell lines displayed alterations in cholesterol and cholesterol ester synthesis that are only evident in the absence of oxysterol (Lagace and et al., 1997). Interestingly there was a slight (40%), but consistent elevation in SM synthesis under the same circumstances, which indicated that OSBP may also regulate basal SM synthesis.

The lack of 25-hydroxycholesterol stimulation in SRD 6 cells is likely due to the inability of OSBP to bind its ligand, or inhibition of activation once oxysterols are bound. *In vitro* OSBP ligand binding is not affected by its phosphorylation status (Ridgway et al., 1997), thus the lack of activation must result from abnormal localization of OSBP to the Golgi apparatus. Perhaps 25-hydroxycholesterol cannot access its binding site when OSBP is at the Golgi apparatus. Thus, only cytosolic OSBP can be activated, and non-ligand bound OSBP that is Golgi localized does not have the ability to activate down-stream events such as the stimulation of SM synthesis. Alternatively, dephosphorylated OSBP bound to the Golgi apparatus may be localized to a region of the apparatus distinct from the site of activation for SM synthesis. Thus, even when ligand is bound it is unable to activate SM synthesis. Evidence for this was seen with the indirect immunofluorescence of OSBP. In sterol-depleted SRD 6 cells, the constitutive Golgi-localized OSBP displayed a more compact fluorescence pattern compared to Golgi-localized OSBP in 25-hydroxycholesterol-treated CHO 7 and SRD 6 cells grown with cholesterol. Thus the OSBP in SRD 6 cells could be segregated in a specific region of the Golgi apparatus, or the structure of the Golgi could be modified.

It is uncertain if the primary effects of oxysterols and OSBP are on sterol metabolism, sphingomyelin metabolism or both. It is feasible that oxysterol simultaneously changes the synthesis of cholesterol and sphingomyelin by altering lipid or protein trafficking at the Golgi apparatus, but more work is needed to test this hypothesis. It should be noted that cholesterol-starved SRD

6 cells and U18666A-treated CHO-K1 cells maintained 25-hydroxycholesterol activation of cholesterol esterification. However, 25-hydroxycholesterol changes ACAT activity by allosteric activation (Chang et al., 1995), in addition to enhanced cholesterol delivery to the ER (Chang et al., 1997). Thus, even though ACAT was stimulated by 25-hydroxycholesterol in SRD 6 cells, this may be distinct from events mediated by oxysterols and OSBP at the Golgi apparatus.

Figure 33 shows the proposed model for the interactions of OSBP localization, and phosphorylation with SM synthesis. In sterol-depleted SRD 6 cells, OSBP is overexpressed, dephosphorylated and constitutively localized to the Golgi. This indicates the possibility of a cholesterol-dependent phosphorylation reaction, possibly at the Golgi apparatus. In support of this hypothesis, the Golgi-enriched fraction of CHO-K1 cells was shown to contain a unique kinase that phosphorylated OSBP (Ridgway et al., 1997). It appears that cycling of OSBP between cellular compartments and OSBP phosphorylation are linked. Under normal conditions OSBP would be rapidly phosphorylated at the Golgi apparatus and released. Thus it would appear predominately in the cytoplasmic/vesicular compartment. This cycle would be interrupted if OSBP was not completely phosphorylated at the Golgi apparatus. If cycling between compartments is blocked, the receptor would be unable to transduce the oxysterol signal.

The finding of OSBP involvement in regulation of SM synthesis at the Golgi has some interesting parallels to studies of a *S. ceravesiae* OSBP homologue,

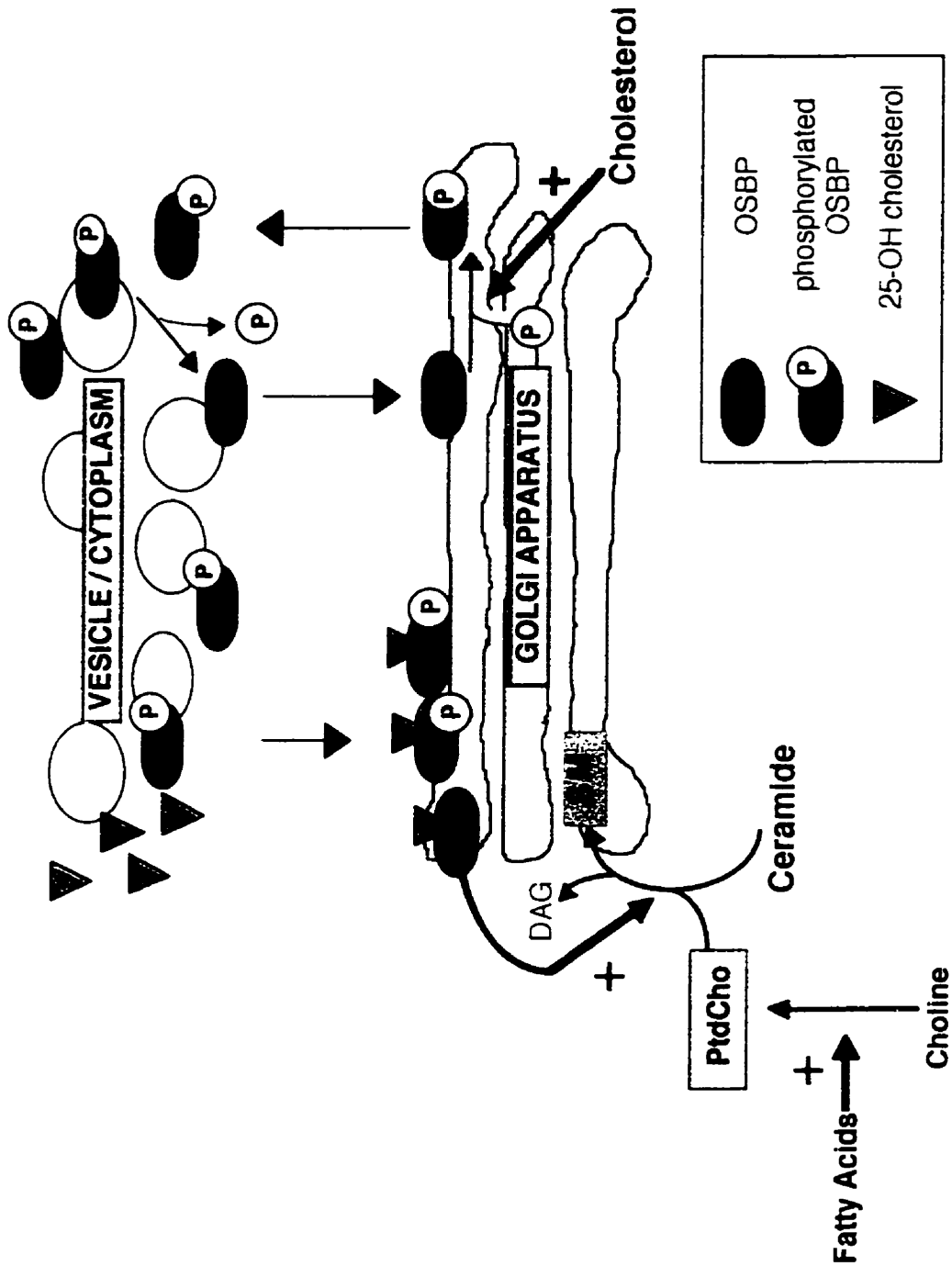

Figure 33. Proposed Model of OSBP regulation and its role in oxysterol-induced SM synthesis

Kes1p, which has been implicated in the regulation of PtdCho metabolism (Fang et al., 1996). Deletion of *KES 1* bypassed a lethal *sec14*^{ts} mutation (Fang et al., 1996). Sec14p is a PtdCho/PtdIns binding protein that regulates PtdCho synthesis and diacylglycerol production (Bankaitis et al., 1989). When PtdCho is bound to Sec14p, PtdCho biosynthesis is due to decreased CT activity (Skinner, et al., 1995). Mutations which inhibit PtdCho synthesis via the CDP-choline pathway also rescue *sec14*^{ts} mutants (Cleves et al. 1991). The results of the lethal *sec14*^{ts} mutation is an accumulation of Golgi PtdCho and the cessation of secretion (Bankaitis et al., 1990). It is thought that Kes1p, which encompasses only the ligand binding domain of OSBP and is a negative regulator of Sec14p, is activated when Golgi diacylglycerol levels are decreased (Fang et al., 1996). It could be proposed that OSBP has a related function by directly or indirectly regulating diacylglycerol and PtdCho levels, possibly through changes in SM synthesis at the Golgi apparatus. How OSBP functions mechanistically in this role has yet to be determined, but it could be possible that OSBP acts as a lipid sensor for cholesterol concentrations at the Golgi.

VI. Summary and Conclusions

The results from this study provide a possible explanation for covariance of PtdCho, SM, and cholesterol in pathological conditions such as atherosclerosis and the Niemann-Pick disorders via coordinate regulation of synthesis and catabolism of these lipids. The important findings of this study are as follows.

The synthesis of PtdCho is regulated by both cholesterol accumulation and deficiency. In both conditions, the regulatory control was shown to occur at the level of CTP:phosphocholine cytidylyltransferase and involve enzyme translocation, activation and, phosphorylation (Shiratori et al., 1995). In sterol-depleted conditions, the main point of regulation appears to be a secondary effect of inactivation of SREBP on fatty acid biosynthesis. In our studies using SRD 2 and SRD 4 cells, which have increased cholesterol synthesis, we did not observe dramatic effects on PtdCho synthesis, only on catabolism.

Regulation of basal and 25-hydroxycholesterol-stimulated SM synthesis is linked to cholesterol metabolism. The point of regulation appears to be altered conversion of ceramide to SM. Basal and oxysterol-stimulated SM synthesis is decreased when cholesterol synthesis is inhibited and cellular cholesterol is depleted. The regulation of SM synthesis by cholesterol and oxysterols may occur through the activation and regulation of OSBP translocation and phosphorylation. Conditions which inhibit oxysterol stimulation of SM synthesis also caused dephosphorylation of OSBP, and in the case of cholesterol auxotrophic SRD 6 cells, this was accompanied by constitutive Golgi

localization of OSBP.

Further work is needed to fully explain the mechanisms involved in metabolic co-regulation of PtdCho, SM and cholesterol. We still need to determine if the link between PtdCho synthesis and catabolism is altered in SRD 6 cells, and determine how SRD 4 PtdCho synthesis is increased. We also need to determine whether biosynthesis or catabolism is primarily affected by the mutations in these cells.

With regard to the role of OSBP in the regulation of lipids, it needs to be determined if altered OSBP regulation affects PtdCho synthesis. Also, we need to investigate further the mechanisms by which OSBP alters SM and cholesterol synthesis. It will also be interesting to identify the kinase involved in cholesterol-stimulated phosphorylation of OSBP, since cholesterol has not been implicated in the regulation of protein kinases.

VII. References

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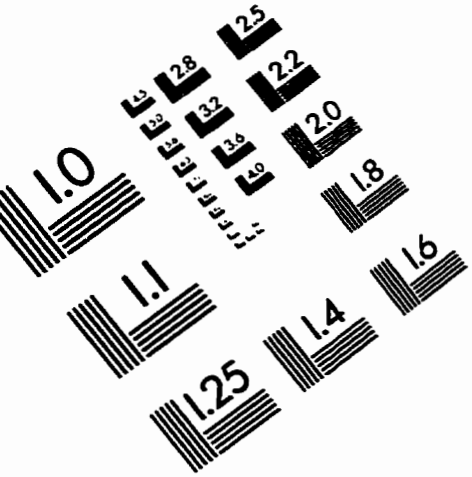

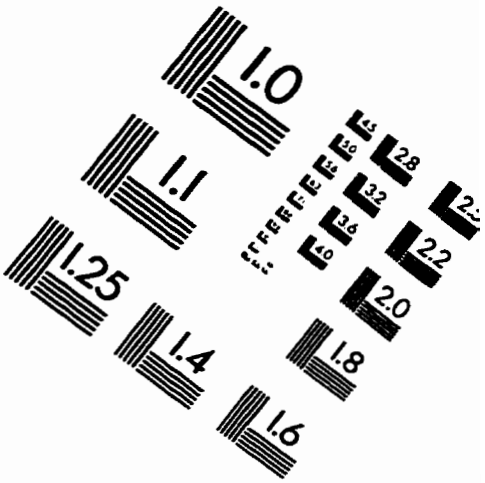

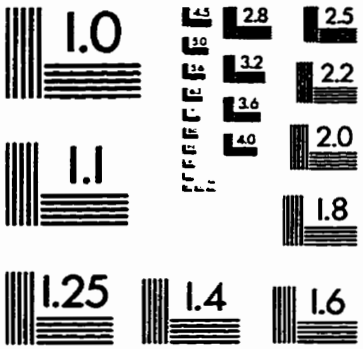

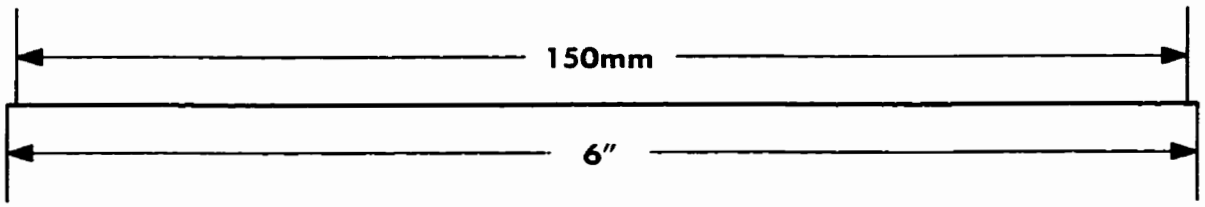

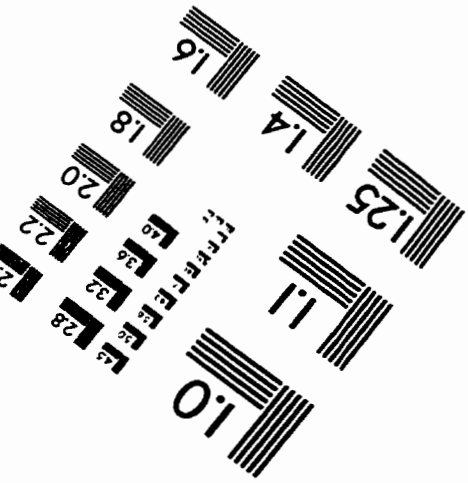

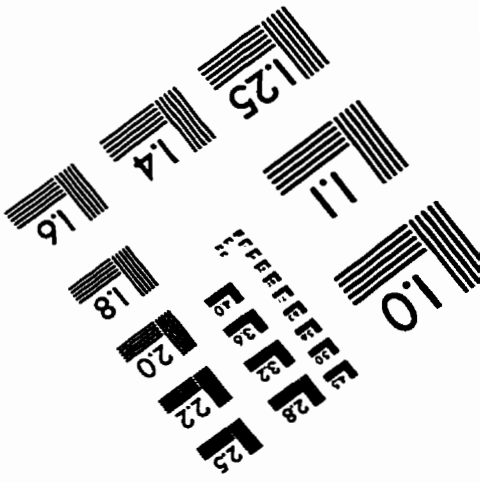

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