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CHAPTER 15

Cholesterol biosynthesis

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1. Introduction

Cholesterol's structure, biosynthetic pathway and metabolic regulation have tested the ingenuity of chemists, biochemists and cell biologists for over 100 years. The last century began with the pioneering work of Heinrich Wieland, who deduced the structure of cholesterol and bile acids, for which Wieland was awarded the Nobel Prize in Chemistry in 1926. How was such a complex molecule synthesized by the cell? Investigation into the cholesterol biosynthetic pathway required the development of isotopic tracer methods in Rudi Schoenheimer's lab in the 1930s. Using these novel techniques, Konrad Bloch and David Rittenberg showed that the ring structure and side chain of cholesterol were derived from acetate, and they identified intermediates in the pathway. Subsequent work by Bloch, John Cornforth and George Popjak succeeded in establishing the biosynthetic origin of all 27 carbons of cholesterol. For his elegant work, Bloch was awarded the Nobel Prize in Chemistry in 1964.

By the 1980s, the cholesterol biosynthetic pathway was understood to be a complex pathway of over 40 cytosolic and membrane-bound enzymes, which was subject to feedback regulation by the end-product, cholesterol, and oxygenated forms (called oxysterols). Genes encoding the key enzymes were cloned, which subsequently revealed the transcriptional and post-translational control of these enzymes. Michael Brown and Joseph Goldstein were awarded the Nobel Prize in Physiology or Medicine in 1985 for their comprehensive work on feedback regulation of cholesterol metabolism. Today, the mechanisms of regulation have been elucidated on a molecular level, although it is still not clear how cholesterol elicits all of the regulation. Furthermore, the evidence is rapidly building that cholesterol's precursors and metabolites might serve as biologically active signaling molecules.

Fig. 1 is an overview of the metabolic and transport pathways that control cholesterol levels in mammalian cells (reviewed in Liscum and Munn [1]). Cholesterol is synthesized from acetyl-CoA via the isoprenoid pathway, and at least four enzymes in the biosynthetic pathway are regulated by cellular cholesterol levels. Essential non-steroidal isoprenoids, such as dolichol, prenylated proteins, heme A and isopentenyl adenosine-containing tRNAs are also synthesized by this pathway. In extrahepatic tissues, most cellular cholesterol is derived from de novo synthesis [2], whereas hepatocytes obtain most of their cholesterol via the receptor-mediated uptake of plasma lipoproteins, such as low-density lipoprotein (LDL). LDL is bound and internalized by the LDL receptor

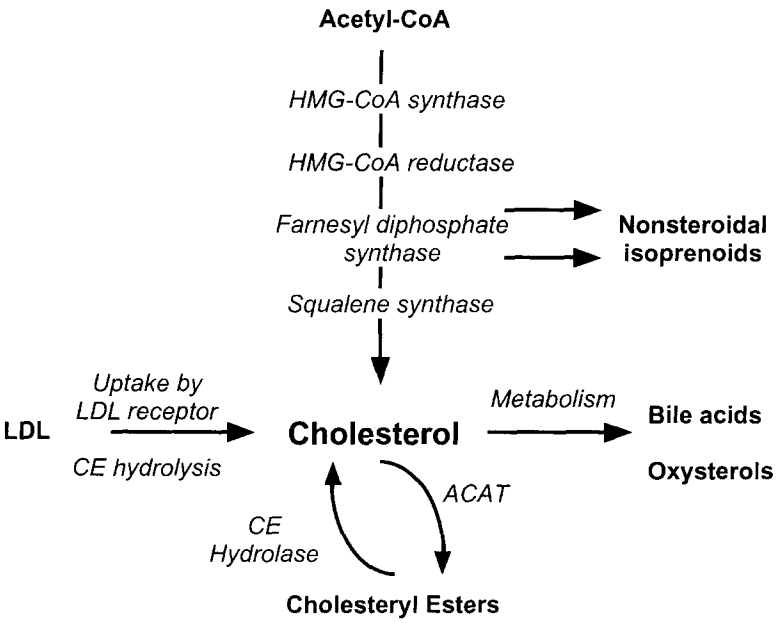

Fig. 1. Overview of the metabolic and transport pathways that control cholesterol levels in mammalian cells. Cholesterol is synthesized from acetyl-CoA and the four key enzymes that regulate cholesterol synthesis are indicated. Cells also obtain cholesterol by uptake and hydrolysis of LDL's cholesteryl esters (CE). End-products derived from cholesterol or intermediates in the pathway include bile acids, oxysterols, cholesteryl esters and non-steroidal isoprenoids. ACAT, acyl-CoA : cholesterol acyltransferase.

and delivered to the acidic late endosomes and lysosomes, where hydrolysis of the core cholesteryl esters occurs (discussed in Chapter 21). The cholesterol that is released is transported throughout the cell. Normal mammalian cells tightly regulate cholesterol synthesis and LDL uptake to maintain cellular cholesterol levels within narrow limits and supply sufficient isoprenoids to satisfy metabolic requirements of the cell. Regulation of cholesterol biosynthetic enzymes takes place at the level of gene transcription, mRNA stability, translation, enzyme phosphorylation and enzyme degradation. Cellular cholesterol levels are also modulated by a cycle of cholesterol esterification by acyl-CoA : cholesterol acyltransferase (ACAT) and hydrolysis of the cholesteryl esters, and by cholesterol metabolism to bile acids and oxysterols.

2. *The cholesterol biosynthetic pathway*

Fig. 2 takes a closer look at the cholesterol biosynthetic pathway, focusing on the enzymes that are regulated, sterol intermediates and the location of enzymes in the cell. Sterols are synthesized from the two-carbon building block, acetyl-CoA. The soluble enzyme acetoacetyl-CoA thiolase interconverts acetyl-CoA and acetoacetyl-CoA, which are then condensed by 3-hydroxy-3-methylglutaryl (HMG)-CoA synthase to form HMG-CoA. There are two forms of HMG-CoA synthase. A mitochondrial

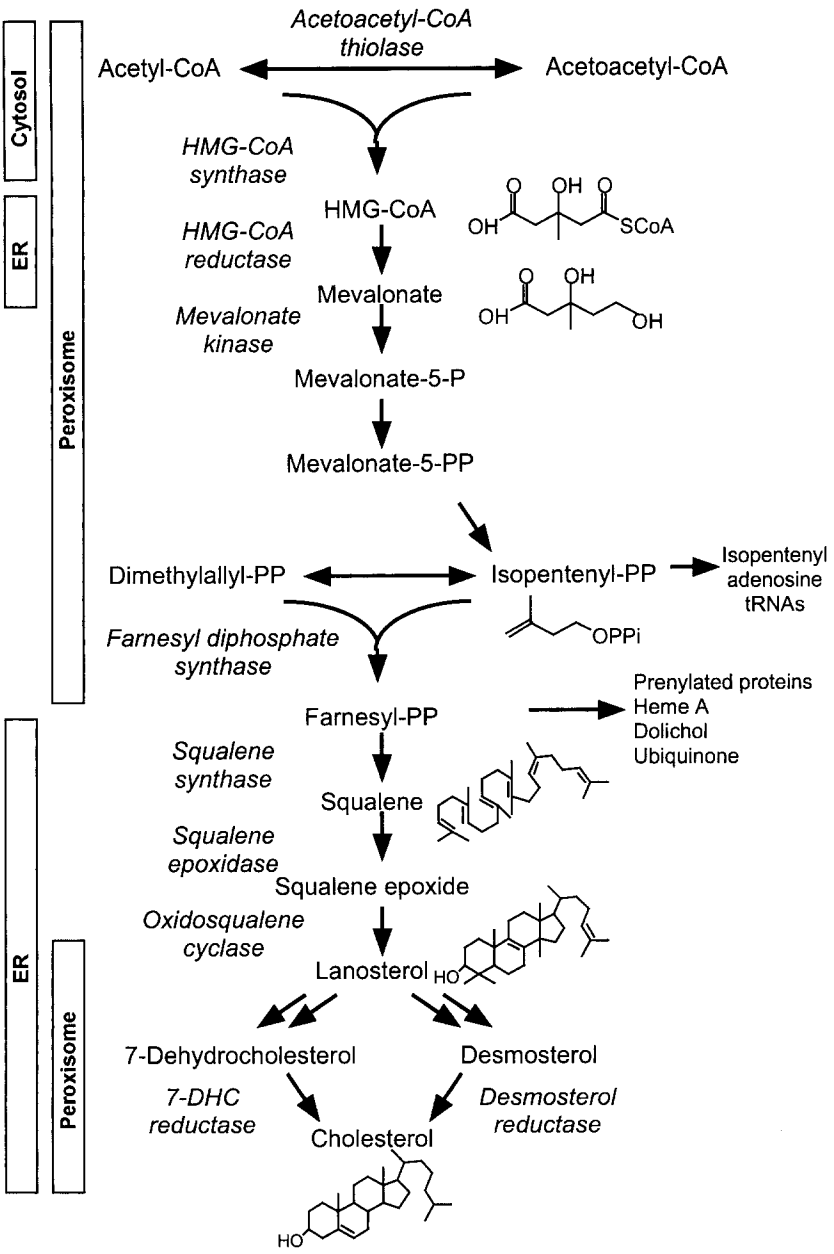

Fig. 2. The cholesterol biosynthetic pathway. Some of the major intermediates and end-products are indicated. Enzymes in the pathway are found in cytosol, endoplasmic reticulum (ER) and peroxisomes, as noted. Figure adapted from Olivier and Krisans [3]. HMG, 3-hydroxy-3-methylglutaryl; DHC, dehydrocholesterol.

form, involved in ketogenesis, predominates in the liver. In extrahepatic tissues, the most abundant form is a soluble enzyme of 53 kDa that is highly regulated by supply of cholesterol (G. Gil, 1986). Like acetoacetyl-CoA thiolase, HMG-CoA synthase has classically been described as a cytosolic enzyme because it is found in the $100,000 \times g$ supernatant of homogenized cells and tissues. However, both enzymes contain peroxisomal targeting sequences [3] and may reside in multiple cellular compartments.

HMG-CoA reductase catalyzes the reduction of HMG-CoA to mevalonate, utilizing two molecules of NADPH. HMG-CoA reductase is a 97-kDa glycoprotein of the endoplasmic reticulum (L. Liscum, 1985) and peroxisomes [3]. Analysis of the endoplasmic reticulum enzyme's domain structure revealed an N-terminal membrane domain with eight transmembrane spans (E.H. Olender, 1992), a short linker, and a C-terminal catalytic domain facing the cytosol (Fig. 3). Transmembrane spans 2–5 share a high degree of sequence similarity with several other key proteins in cholesterol metabolism; this region is termed the sterol-sensing domain (described in Section 3.5). Elucidation of the crystal structure of the HMG-CoA reductase catalytic domain indicated that the active protein is a tetramer [4], which is consistent with biochemical analysis. The monomers appear to be arranged in two dimers, with the active sites at the monomer–monomer interface. The dimer–dimer interface is predominantly hydrophobic.

HMG-CoA reductase is the rate-determining enzyme of the cholesterol biosynthetic pathway and, like HMG-CoA synthase, is highly regulated by supply of cholesterol. Thus, the enzyme has received intense scrutiny as a therapeutic target for treatment of hypercholesterolemia. The enzyme is inhibited by a class of pharmacological agents, generally called statins, which have an HMG-like moiety and a bulky hydrophobic group [5] (Fig. 4). Statins occupy the HMG-binding portion of the active site, preventing HMG-CoA from binding (E.S. Istvan, 2001). Also, the bulky hydrophobic group causes disordering of several catalytic residues. Thus, statins are potent, reversible competitive inhibitors of HMG-CoA reductase with K_i values in the nanomolar range. Elevated plasma cholesterol levels are a primary risk factor for coronary artery disease, and statin inhibition of HMG-CoA reductase effectively reduces cholesterol levels and decreases overall mortality. However, complete inhibition of HMG-CoA reductase by statins will kill cells, even if exogenous cholesterol is supplied. That is because complete inhibition deprives cells of all mevalonate-derived products, including essential non-steroidal isoprenoids. To survive, cells must produce a small amount of mevalonate that, when limiting, is used preferentially by higher affinity pathways for non-steroidal isoprenoid production (S. Mosley, 1983).

Mevalonate is metabolized to farnesyl-diphosphate (-PP) by a series of enzymes localized in peroxisomes. First, mevalonate kinase phosphorylates the 5-hydroxy group of mevalonic acid. The enzyme is a homodimer of 40 kDa that is subject to feedback inhibition by several isoprenoid intermediates [6]. Mutations in the mevalonate kinase gene lead to the human genetic disease mevalonic aciduria (discussed in Section 2.2). The product of mevalonate kinase, mevalonate-5-P, is then phosphorylated to form mevalonic acid-5-PP, which is decarboxylated and dehydrated by mevalonate-PP decarboxylase to form isopentenyl-PP. Isopentenyl-PP is in equilibrium with its isomer, dimethylallyl-PP. Farnesyl-PP synthase catalyzes the head to tail condensations of two molecules of isopentenyl-PP with dimethylallyl-PP to form farnesyl-PP. The

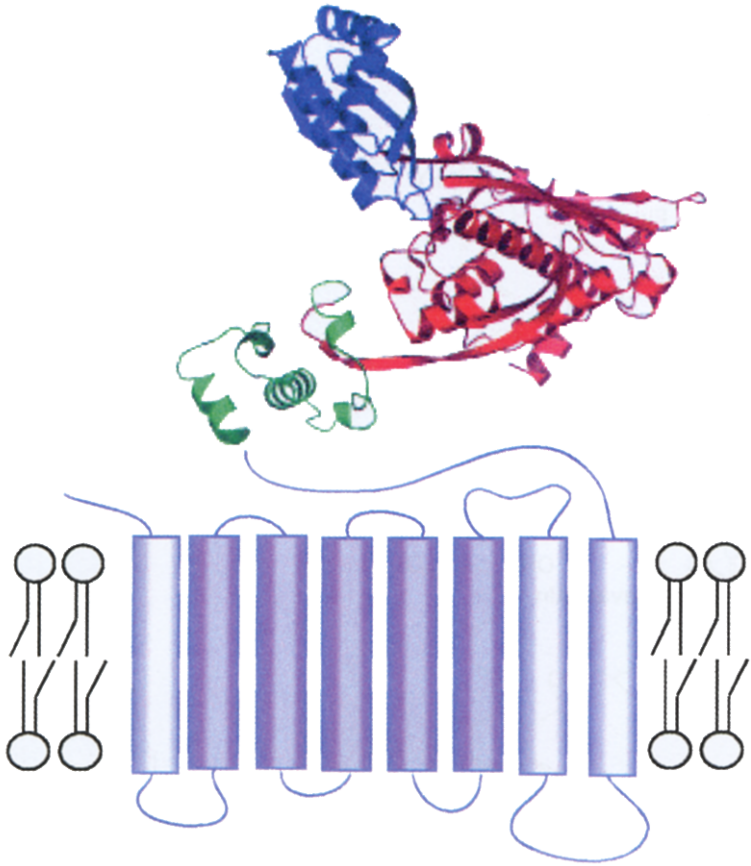

Fig. 3. Domain structure of the endoplasmic reticulum HMG-CoA reductase. The crystal structure of the catalytic domain has been determined and is depicted as a ribbon diagram (courtesy of Eva S. Istvan, Washington University School of Medicine). The catalytic domain consists of a small helical domain (green), a large central element resembling a prism (red), which contains the HMG-CoA-binding site, and a small domain to which NADPH binds (blue) [4]. The structure of the membrane domain has not been solved; however, it is known that eight transmembrane spans embed the protein into the endoplasmic reticulum membrane. Spans 2–5 (darker cylinders) are termed the sterol-sensing domain and mediate the regulated degradation of the enzyme.

enzyme is part of a large family of prenyltransferases that synthesize the backbones for all isoprenoids, including cholesterol, steroids, prenylated proteins, heme A, dolichol, ubiquinone, carotenoids, retinoids, chlorophyll and natural rubber (K.C. Wang, 2000).

Squalene synthase is a 47-kDa protein of the endoplasmic reticulum and catalyzes the first committed step in cholesterol synthesis. The enzyme condenses two molecules of farnesyl-PP and then reduces the presqualene-PP intermediate to form squalene. A large N-terminal catalytic domain faces the cytosol, anchored to the membrane by a C-terminal domain. This orientation may allow the enzyme to receive the hydrophilic substrates from the cytosol and release the hydrophobic product into the endoplasmic

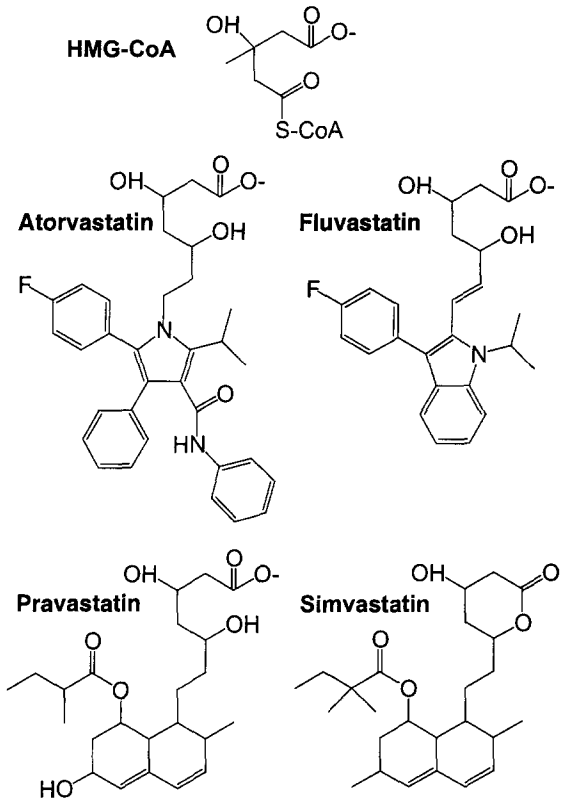

Fig. 4. Chemical structures of HMG-CoA and several statin inhibitors of HMG-CoA reductase. Atorvastatin (Lipitor), fluvastatin (Lescol), pravastatin (Pravachol) and simvastatin (Zocor) are widely prescribed cholesterol-lowering drugs.

reticulum membrane for further metabolism [7]. Squalene synthase is highly regulated by the cholesterol content of the cell. Thus, it plays an important role in directing the flow of farnesyl-PP into the sterol or non-sterol branches of the pathway (M.S. Brown, 1980) [7].

Squalene is converted into the first sterol, lanosterol, by the action of squalene epoxidase and oxidosqualene cyclase. Lanosterol is then converted to cholesterol by a series of oxidations, reductions, and demethylations. The required enzyme reactions have been defined and metabolic intermediates identified; however, the precise sequence of reactions between lanosterol and cholesterol remains to be established [8] (Fig. 5). There is evidence for two alternative pathways that differ in when the $\Delta 24$ double bond is reduced (discussed in Section 2.3). Both 7-dehydrocholesterol and desmosterol have been postulated to be the immediate precursor of cholesterol. One of the key enzymes in the latter part of the pathway is 7-dehydrocholesterol $\Delta 7$-reductase, a 55-kDa integral membrane protein. Mutations in the gene for 7-dehydrocholesterol $\Delta 7$-reductase cause the human genetic disease Smith–Lemli–Opitz syndrome (discussed in Section 2.3).

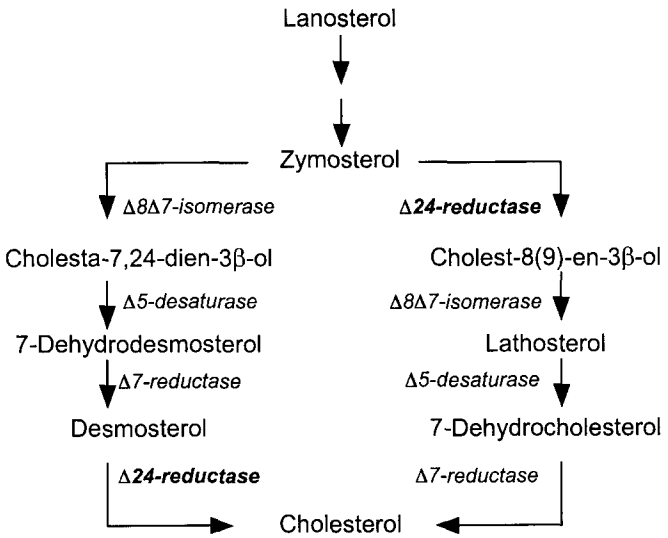

Fig. 5. Final steps in the cholesterol biosynthetic pathway. Alternate steps have been proposed for the conversion of zymosterol to cholesterol, which differ in when the Δ24-reductase reaction occurs. Figure adapted from Waterham and Wanders [8] and Kelly and Hennekam [11].

2.1. Enzyme compartmentalization

Where does cholesterol synthesis take place? All of the enzymes that convert acetyl-CoA to farnesyl-PP have classically been thought of as cytosolic enzymes, with the exception of HMG-CoA reductase, which is typically depicted as an endoplasmic reticulum enzyme with the catalytic site facing the cytosol. Enzymes that convert farnesyl-PP to cholesterol are classically described as microsomal. However, there is now strong evidence that all but one of these enzymes is also, or exclusively, peroxisomal [3]. The molecular cloning of cDNAs encoding many of these enzymes has revealed peroxisomal targeting sequences. The availability of antibodies has allowed immunocytochemical localization to peroxisomes. Together these data suggest that peroxisomes may play an active role in all steps in the cholesterol biosynthetic pathway except the conversion of farnesyl-PP to squalene, which is catalyzed by squalene synthase found solely in the endoplasmic reticulum.

HMG-CoA reductase is the one exception to the rule. Immunocytochemistry and immunoblotting have localized HMG-CoA reductase to both the endoplasmic reticulum and peroxisomes; however, no peroxisomal targeting motif has been found in the HMG-CoA reductase protein sequence. Furthermore, the peroxisomal HMG-CoA reductase has an apparent molecular weight of 90 kDa whereas the endoplasmic reticulum enzyme is 97 kDa (W.H. Engfelt, 1997). The peroxisomal enzyme exhibits other distinct properties: it is resistant to statin inhibition, the enzyme's activity is not regulated by phosphorylation, the protein's turnover is not regulated by mevalonate. Altogether, this evidence suggests that the endoplasmic reticulum and peroxisome enzymes are functionally and structurally distinct (N. Aboushadi, 2000).

Additional evidence for the involvement of peroxisomes in cholesterol biosynthesis comes from analysis of diseases of peroxisomal deficiency. Zellweger syndrome, neonatal adrenoleukodystrophy, and infantile Refsum's disease are all diseases of peroxisome biogenesis [9]. In most of these peroxisomal disorders, the peroxisomal matrix proteins are synthesized in the cytosol as normal, but they cannot be assembled into nascent peroxisomes due to mutations in one of at least 12 different genes encoding proteins necessary for peroxisomal protein targeting and import. Fibroblasts from individuals with peroxisome biogenesis disorders show reduced enzymatic activities of cholesterol biosynthetic enzymes, reduced levels of cholesterol synthesis and lower cholesterol content [3]. These data support the hypothesis that part of the cholesterol synthesis pathway is peroxisomal.

It is not clear why cholesterol synthesis is compartmentalized and requires intermediates to cycle between peroxisomes and the cytosol. It is also unclear why some of the enzymes are found in multiple compartments and others are solely in endoplasmic reticulum or peroxisomes. As noted, cholesterol synthesis is a very complex process and compartmentalization may represent another level of regulation [3].

2.2. Mevalonic aciduria

Cholesterol synthesis is essential for normal development and maintenance of tissues that cannot obtain cholesterol from plasma lipoproteins, such as brain. Furthermore, the biosynthetic pathway supplies non-steroidal isoprenoids that are required by all cells. Thus, it is not surprising that metabolic defects in the cholesterol biosynthetic pathway have devastating consequences.

The first recognized human metabolic defect in the biosynthesis of cholesterol and isoprenoids was mevalonic aciduria [10]. Mevalonic aciduria is an autosomal recessive disorder that is quite rare, with only 19 known patients. In normal individuals, a small amount of mevalonic acid diffuses into the plasma at levels proportional to the rate of cellular cholesterol formation. Patients with mild mevalonic aciduria excrete 3000–6000 times the normal amount of mevalonic acid and patients with the severe form of the disease excrete 10,000–200,000 times the normal amount. Enzyme assays using cell lysates showed that mevalonate kinase activity was markedly deficient in patient samples and genetic analysis has revealed nucleotide changes in the mevalonate kinase gene that lead to amino acid substitutions. Because of this enzyme deficiency, there is little to no feedback inhibition of HMG-CoA reductase and, thus, mevalonate is overproduced.

Clinical features of mevalonic aciduria include failure to thrive, anemia, gastroenteropathy, hepatosplenomegaly, psychomotor retardation, hypotonia, ataxia, cataracts, and dysmorphic features [10]. Surprisingly, patients with severe deficiencies in mevalonate kinase show normal plasma cholesterol levels and cultured mevalonic aciduria fibroblasts show rates of cholesterol synthesis half that of normal cells. Close examination of cholesterogenic enzymes in mevalonic aciduria fibroblasts has revealed a 6-fold increase in HMG-CoA reductase activity, which is postulated to compensate for the low mevalonate kinase activity.

2.3. Smith–Lemli–Opitz syndrome

A second metabolic defect in cholesterol synthesis leads to Smith–Lemli–Opitz syndrome (SLOS) (B.U. Fitzkey, 1999) [11]. SLOS is a relatively common autosomal recessive disorder, with estimates of incidence ranging from 1 in 10,000 to 1 in 60,000. Four lines of evidence pointed to the metabolic defect in SLOS patients. (1) Individuals with SLOS were found to have markedly elevated levels of plasma 7-dehydrocholesterol and low plasma cholesterol levels. (2) 7-Dehydrocholesterol Δ7-reductase activity was deficient in SLOS patient samples and the amount of residual activity could be correlated with severity of the disease. (3) Rodents treated with AY-9944, an inhibitor of 7-dehydrocholesterol Δ7-reductase, developed SLOS-like malformations [11]. (4) Cloning of the gene for 7-dehydrocholesterol Δ7-reductase led to identification of a splice-site mutation and amino acid substitutions in SLOS patients.

Severely reduced cholesterol synthesis is predicted to have severe consequences on development of the fetus because cholesterol is only obtained from the maternal circulation during the first trimester [11]. In addition, the brain is predicted to be severely affected because plasma lipoproteins cannot cross the blood–brain barrier and most, if not all, cholesterol needed for brain growth is synthesized locally (S.D. Turley, 1998) [2,12]. Indeed, severely affected SLOS infants who died soon after birth were found to have functionally null 7-dehydrocholesterol Δ7-reductase alleles [12], whereas typical affected individuals likely have some residual 7-dehydrocholesterol Δ7-reductase catalytic activity.

Patients with SLOS have mental retardation and microcephaly, which is consistent with cholesterol synthesis being required for normal brain development. Clinical features also include failure to thrive, and characteristic craniofacial, skeletal and genital anomalies. The clinical phenotype appears to be due to a lack of cholesterol rather than the cellular accumulation of 7-dehydrocholesterol (W. Gaoua, 2000). A recent multicenter clinical trial has shown that SLOS children fed a diet supplemented with cholesterol show improved growth and neurodevelopment (i.e. language and cognitive skills) (M. Irons, 1997; E.R. Elias, 1997). It is likely that the diet fulfilled the daily requirement for cholesterol and down-regulated endogenous 7-dehydrocholesterol synthesis.

What are the final steps in the cholesterol biosynthetic pathway? SLOS may provide an answer to that question. As noted above, there is evidence for two alternative pathways, which differ in when the Δ24 double bond is reduced [11]. In both pathways, lanosterol is demethylated to form zymosterol (Fig. 5). Then, zymosterol can be metabolized sequentially by a Δ24-reductase, Δ8,Δ7-isomerase, and Δ5-desaturase to form 7-dehydrocholesterol, which is reduced at the Δ7 position to form cholesterol. Alternatively, zymosterol can be metabolized by the Δ8,Δ7-isomerase and Δ5-desaturase, to form 7-dehydrodesmosterol. 7-Dehydrodesmosterol is metabolized by the Δ7-reductase to form desmosterol and then by the Δ24-reductase to form cholesterol. The fact that the SLOS deficiency in Δ7-reductase leads to a buildup of 7-dehydrocholesterol rather than 7-dehydrodesmosterol is interpreted to mean that the former pathway is the principal one. However, the latter pathway must also be used because desmosterol is an abundant cholesterol precursor in certain tissues. It has been suggested that the final steps in the biosynthetic pathway may be tissue specific.

Table 1
Inborn errors of sterol biosynthesis

Syndrome	Metabolic defect
Mevalonic aciduria	Mevalonate kinase
Smith–Lemli–Opitz	Sterol Δ7-reductase
Desmosterolosis	Sterol Δ24-reductase
Rhizomelic chondrodysplasia punctata (CDP)	Pex7 peroxisomal enzyme import
CDP X-linked dominant (CDPX2)	Sterol Δ8,Δ7-isomerase
CHILD syndrome (congenital hemidysplasia with ichthyosis and limb defects)	Sterol Δ8,Δ7-isomerase Sterol C-4 demethylase
Greenberg skeletal dysplasia	Sterol Δ14-reductase

These syndromes and their corresponding metabolic defects are reviewed in Kelley [13].

Perhaps, in SLOS cells, any 7-dehydrodesmosterol that accumulates is metabolized by the available Δ24-reductase to form 7-dehydrocholesterol.

2.4. Other enzyme deficiencies

Other inborn errors of sterol biosynthesis have been reviewed by Kelley [13] and are summarized in Table 1. Rhizomelic chondrodysplasia punctata, like Zellweger syndrome, exhibits defective sterol synthesis due to the lack of key peroxisomal enzymes of cholesterol biosynthesis. CDPX2, also known as Conradi–Hünermann syndrome, and most cases of CHILD syndrome are due to mutations in the sterol Δ8,Δ7-isomerase gene, which is located on the X chromosome. Mutations in a single gene may lead to different syndromes with similar, but distinct, pathologies due to the mosaicism of X-chromosome inactivation. A few cases of CHILD syndrome may be due to mutations in the sterol C-4 demethylase gene, also located on the X chromosome.

3. Regulation of cholesterol synthesis

Isoprenoid synthesis is regulated by the sterol end-product of the biosynthetic pathway, by non-sterol intermediates, and also by physiological factors. The cholesterol content of the cell controls several enzymes in the biosynthetic pathway, but the focus has been on the rate-limiting enzyme, HMG-CoA reductase. Different regulators have different mechanisms of action. For example, sterols have been shown to regulate at the level of HMG-CoA reductase transcription whereas non-sterols regulate HMG-CoA reductase mRNA translation. Both sterols and non-sterols are needed for regulation of HMG-CoA reductase protein degradation [14]. Physiological factors that influence cholesterol synthesis include diurnal rhythm, insulin and glucagon, thyroid hormone, glucocorticoids, estrogen and bile acids [15]. These factors regulate HMG-CoA reductase by transcriptional, translational and post-translational mechanisms.

3.1. Transcriptional regulation

HMG-CoA reductase is the rate-limiting enzyme in the cholesterol biosynthetic pathway and combined regulation of HMG-CoA reductase synthesis and turnover can alter steady state levels of the enzyme 200-fold. HMG-CoA reductase is regulated in parallel with at least three other enzymes in the cholesterol biosynthetic pathway, HMG-CoA synthase, farnesyl-PP synthase and squalene synthase, as well as the LDL receptor. This coordinate regulation is due to the fact that each gene has a similar sequence (*cis*-acting element) within the promoter that recognizes a common *trans*-acting transcription factor. Availability of the transcription factor to bind to the promoter sequence is influenced by the cellular cholesterol content.

Fig. 6 illustrates the current model of cholesterol-mediated transcriptional regulation [16–19]. The 5′ flanking regions of cholesterol-regulated genes have one to three copies of a 10-bp non-palindromic nucleotide sequence termed the sterol regulatory element (SRE). SREs are conditional positive elements that are required for gene transcription in cholesterol-depleted cells. The SRE sequence found in the LDL receptor gene is 5′-ATCACCCCAC-3′. SREs have been identified in the HMG-CoA synthase, HMG-CoA reductase, farnesyl-PP synthase and squalene synthase genes, as well as genes of fatty acid synthesis. However, there is not a strict SRE consensus sequence and identifying functional SREs has been difficult [17].

The transcription factor that binds the SRE is termed the SRE-binding protein (SREBP) [16,20,21]. The first SREBP to be identified was the protein that bound to the LDL receptor promoter (M.R. Briggs, 1990; X. Wang, 1990). Cloning of SREBP cDNAs (C. Yokoyama, 1993; X. Hua, 1993) revealed that there are two SREBP genes that produce three distinct proteins. SREBP-1a and -1c are derived from one gene that contains two promoters and differ in the length of the N-terminal transactivation domain. SREBP-2 is derived from a second gene and is 45% identical to SREBP-1a. SREBP-1c is the predominant isoform in liver and adipocytes. It was isolated independently and called adipocyte determination and differentiation-dependent factor 1 (P. Tontonoz, 1993). Here when the term SREBP is used, the information is relevant for all three isoforms.

SREBPs are cytosolic 68-kDa proteins with a canonical basic helix–loop–helix leucine zipper (bHLH-Zip) motif that is present in other transcription factors. Unlike other bHLH-Zip transcription factors, SREBPs have a tyrosine in place of a conserved arginine, which allows them to bind to the inverted E-box motif (5′-CANNTG-3′) in addition to SREs (J.B. Kim, 1995). By binding to SREs, SREBPs coordinately regulate multiple enzymes involved in fatty acid synthesis and lipogenesis [20,21].

An additional feature that distinguishes SREBPs from other bHLH-Zip transcription factors is that SREBP genes encode 125-kDa membrane proteins that are inserted into the endoplasmic reticulum and serve as precursors for the active transcription factors. SREBPs have three functional domains: an N-terminal 68-kDa fragment containing the bHLH-Zip transcription factor, two membrane-spanning segments, and a C-terminal regulatory domain. It is the sequential two-step cleavage of the full-length precursor SREBP and release of the 68-kDa N-terminal bHLH-Zip domain that is influenced by the cellular cholesterol content.

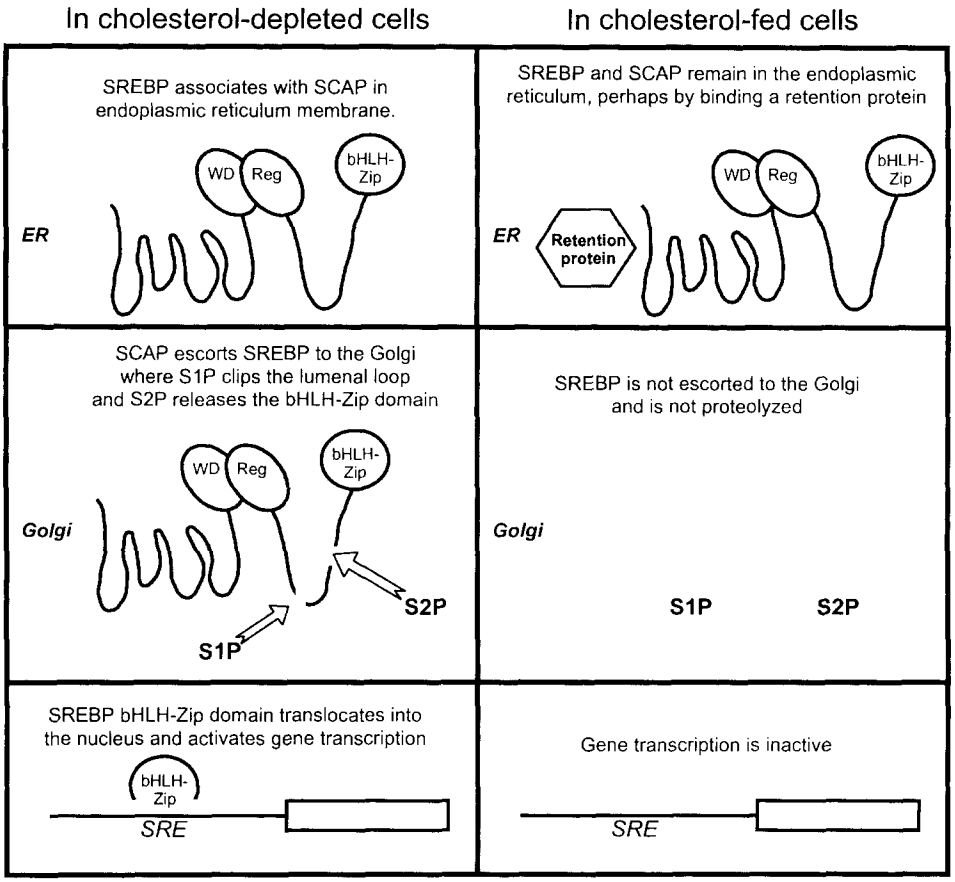

Fig. 6. Current model of cholesterol regulation of SREBP proteolysis. The sterol regulatory element-binding protein (SREBP) precursor is inserted into the endoplasmic reticulum (ER) membrane. The SREBP regulatory domain (Reg) interacts with the SREBP cleavage-activating protein (SCAP), likely through SCAP's WD repeats. When cholesterol levels are low, SCAP escorts SREBP to the Golgi where the bHLH-ZIP domain is released by site-1 protease (S1P) cleavage of a lumenal loop followed by site-2 protease (S2P) cleavage within a transmembrane span. The mature SREBP translocates into the nucleus and activates gene transcription. In cholesterol replete cells, the SREBP precursor and SCAP remain in the endoplasmic reticulum and the SREBP precursor is not proteolyzed to release the bHLH-Zip transcription factor.

Identification of the proteins required for regulated SREBP cleavage was accomplished using somatic cell genetic approaches. Mutant Chinese hamster ovary cells with abnormal regulation of cholesterol and fatty acid metabolism, which were selected over the past 20 years, proved invaluable for this goal [22]. Using expression cloning strategies, genes were isolated that restored SREBP-mediated transcription in each mutant. This work led to identification of two proteases and an escort protein required for SREBP precursor cleavage. Cleavage of the SREBP precursor at site 1 requires a subtilisin-like serine protease (J. Sakai, 1998), whereas cleavage at site 2 requires a zinc metalloprotease (R.B. Rawson, 1997). Transcription factor release is controlled

by a chaperone, SREBP cleavage-activating protein (SCAP), which escorts the SREBP precursor to the Golgi where the proteases reside (X. Hua, 1996).

How is SREBP proteolysis controlled by cholesterol? A hint that this event may involve vesicle trafficking came from the finding that the SREBP precursor's *N*-linked carbohydrates are endoglycosidase-H-resistant (trimmed by Golgi mannosidases) when cellular cholesterol levels are low, and endoglycosidase-H-sensitive when cellular cholesterol levels are high (A. Norturfft, 1998, 1999). Thus, release of the mature SREBP transcription factor appeared to coincide with transport to the Golgi. The current model is as follows (Fig. 6). When cellular cholesterol levels are low, the SREBP precursor is synthesized and inserted into the endoplasmic reticulum membrane. The C-terminal regulatory domain of the SREBP precursor interacts with the C-terminus of SCAP, likely through SCAP's four WD repeats (J. Sakai, 1998). SCAP and the SREBP precursor are then transported to the Golgi where the lumenal loop of the SREBP precursor is clipped by the site-1 protease; however, the two halves of the protein remain membrane-anchored. Then the site-2 protease clips the N-terminal SREBP intermediate within the first membrane-spanning segment, releasing the soluble transcription factor, mature SREBP. Upon translocation to the nucleus, the mature SREBP binds SRE sequences within the promoters of target genes and enhances their transcription.

When cellular cholesterol levels rise to a threshold level, SCAP and the SREBP precursor no longer travel to the Golgi and the SREBP precursor is not proteolyzed to produce the mature SREBP. As a result, transcription of target genes declines to basal levels. Evidence for SCAP–SREBP transport is two-fold. The sterol-dependent movement of SCAP has been directly visualized in cultured cells transfected with a green fluorescent protein–SCAP fusion protein (A. Nohturfft, 2000). Furthermore, in vitro vesicle-budding assays have demonstrated that oxysterols suppress SREBP–SCAP complexes from entering into vesicles budding from the endoplasmic reticulum.

The mechanism by which SCAP senses cellular cholesterol is not known. Sensing is postulated to involve a five-transmembrane segment of SCAP with sequence similarity to a five-transmembrane segment of HMG-CoA reductase, called the sterol-sensing domain (discussed in Section 3.5). Consistent with this hypothesis, several constitutively active SCAP mutants have been isolated that have point mutations in the sterol-sensing domain (X. Hua, 1996). Also, there is recent evidence that SCAP interacts with a protein that is retained in the endoplasmic reticulum (T. Yang, 2000). Therefore, control of SCAP and SREBP transit to the Golgi could depend upon sterol-dependent binding of SCAP's sterol-sensing domain to a retention protein.

SREBPs control not only cholesterol synthesis, but also the synthesis of fatty acids (via fatty acid synthase, acetyl-CoA carboxylase, and stearoyl-CoA desaturase 2), triacylglycerols and phospholipids (glycerol-3-phosphate acyltransferase). This was illustrated most dramatically in transgenic mice overexpressing the nuclear forms of SREBP-1a, -1c and -2 in liver [20]. Overexpression of each isoform resulted in activation of a full spectrum of cholesterol and fatty acid biosynthetic enzymes; however, absolute levels of induction of each enzyme and the subsequent liver phenotype varied according to the isoform expressed. From these data, the following conclusions can be drawn. SREBP-1a is a strong activator of cholesterol and fatty acid synthesis and is likely to be important in rapidly dividing cells that require lipid for membrane production.

SREBP-1c predominates in the liver, where it primarily activates genes of fatty acid synthesis. SREBP-1c appears to be important for maintaining basal transcription levels of fatty acid and cholesterol biosynthetic enzymes during periods of fasting. It also plays a role in the insulin response [21]. SREBP-2 selectively activates cholesterol biosynthetic genes and the LDL receptor, and primarily responds when the liver's demand for cholesterol rises.

3.2. mRNA translation

HMG-CoA reductase is also subject to translational control by a mevalonate-derived non-sterol regulator (D. Peffley, 1985; M. Nakanishi, 1988). This component of the regulatory mechanism can only be observed when cultured cells are acutely incubated with statins, which block mevalonate formation. Under those conditions, sterols have no effect on HMG-CoA reductase mRNA translation; however, mevalonate reduces the HMG-CoA mRNA translation by 80% with no change in mRNA levels. Translational control of hepatic HMG-CoA reductase by dietary cholesterol was shown in an animal model in which polysome-associated HMG-CoA reductase mRNA was analyzed in cholesterol-fed rats (C.M. Chambers, 1997). It was found that cholesterol feeding increased the portion of mRNA associated with translationally inactive monosomes and decreased the portion of mRNA associated with translationally active polysomes. The mechanism of HMG-CoA reductase translational control has not been elucidated.

3.3. Phosphorylation

Many key metabolic enzymes are modulated by phosphorylation–dephosphorylation and it has long been known that HMG-CoA reductase catalytic activity is inhibited by phosphorylation (Z.H. Beg, 1973). Rodent HMG-CoA reductase is phosphorylated on Ser 871 by an AMP-activated protein kinase that uses ATP as a phosphate donor (P.R. Clarke, 1990). However, examination of HMG-CoA reductase activity in rat liver showed that phosphorylation–dephosphorylation could not account for the long-term regulation that occurred with diurnal light cycling, fasting, or cholesterol-supplemented diet (M.S. Brown, 1979). Approximately 75–90% of HMG-CoA reductase enzyme was found to be phosphorylated (inactive) under all physiological conditions. This reservoir of inactive enzyme may allow cells to respond transiently to short-term cholesterol needs.

The AMP-activated kinase that phosphorylates and inactivates HMG-CoA reductase also phosphorylates and inactivates acetyl-CoA carboxylase. It has been suggested that, when cellular ATP levels are depleted causing AMP levels to increase, the resultant activation of the kinase would inhibit cholesterol and fatty acid biosynthetic pathways, thus conserving energy (D.G. Hardie, 1992). Consistent with this hypothesis, cholesterol synthesis was reduced when ATP levels were depleted by incubation with 2-deoxy-D-glucose (R. Sato, 1993). However, cholesterol synthesis was not reduced when ATP levels declined in cells expressing a Ser 871 to Ala mutant form of HMG-CoA reductase, which is not phosphorylated (R. Sato, 1993). Therefore, HMG-CoA reductase phosphorylation appears to be important for preserving cellular energy stores rather than end-product feedback regulation.

3.4. Proteolysis

Raising the cellular cholesterol content not only stops transcription of the genes encoding the cholesterol biosynthetic enzymes, but it also leads to accelerated degradation of the rate-limiting enzyme, HMG-CoA reductase. In cholesterol-depleted cells, HMG-CoA reductase is a stable protein that is degraded slowly ($t_{1/2} = 13$ h) (J.R. Faust, 1982). If cholesterol repletion simply stopped transcription of the HMG-CoA reductase gene, that would lead to a slow decline in HMG-CoA reductase enzyme activity owing to stability of the protein; however, in the presence of excess sterols or mevalonate there is rapid ($t_{1/2} = 3.6$ h) (J.R. Faust, 1982) and selective degradation of the enzyme, which results in more precise control of cellular sterol synthesis.

The HMG-CoA reductase membrane domain is necessary and sufficient for regulated degradation. Expression of the cytosolic catalytic domain results in a stable protein that is not subject to regulated degradation (G. Gil, 1985), whereas expression of the N-terminal membrane domain linked to a reporter protein results in regulated degradation of the reporter (D. Skalnik, 1988). Despite the complex topology of HMG-CoA reductase, no proteolytic intermediates have ever been detected. How is HMG-CoA reductase proteolyzed? Possibilities include vesicular translocation to lysosomes or autophagy of HMG-CoA reductase-containing endoplasmic reticulum membranes; however, the degradation appears to be rapid and selective for HMG-CoA reductase. It is also unaffected by inhibitors of protein transport through the Golgi (K. Chun, 1990). Another possibility is HMG-CoA reductase digestion by resident endoplasmic reticulum proteases. Subcellular fractionation and use of specific protease inhibitors has revealed that HMG-CoA reductase degradation occurs in purified endoplasmic reticulum membranes and is inhibited by lactacystin, an inhibitor of the proteasome (T. McGee, 1996). Additional evidence that the proteosome is involved comes from study of the yeast ortholog, Hmg2p, which is subject to regulated degradation like the mammalian enzyme. A yeast genetic approach has led to the identification of three proteins that are involved in the reverse translocation of endoplasmic reticulum proteins and disposal by the proteosome (R.Y. Hampton, 1996) [23]. Evidence that HMG-CoA reductase is polyubiquitinated prior to proteolysis has been provided for the yeast (R.Y. Hampton, 1997) and mammalian (T. Ravid, 2000) enzymes.

What is the signal for accelerated HMG-CoA reductase degradation? In mammalian cells, both mevalonate-derived non-sterols and sterols are required. That is, in cholesterol-depleted cells, the addition of sterols leads to accelerated HMG-CoA reductase degradation only when the sterols are accompanied by a mevalonate-derived non-sterol signal (M. Nakanishi, 1988; J. Roitelman, 1992; T.E. Meigs, 1997). Regulated degradation has also been demonstrated through in vitro experiments. HMG-CoA reductase degradation is more rapid in endoplasmic reticulum membranes isolated from mevalonate- or sterol-treated cells (T. McGee, 1996) and in hepatic microsomes prepared from mevalonate-treated rats (C. Correll, 1994). Both the sterol and non-sterol signals are blocked by cycloheximide, indicating that regulated turnover of HMG-CoA reductase requires ongoing protein synthesis (K. Chun, 1990; J. Roitelman, 1992; T. Ravid, 2000).

There is strong evidence that the non-sterol isoprenoid signal for HMG-CoA reduc-

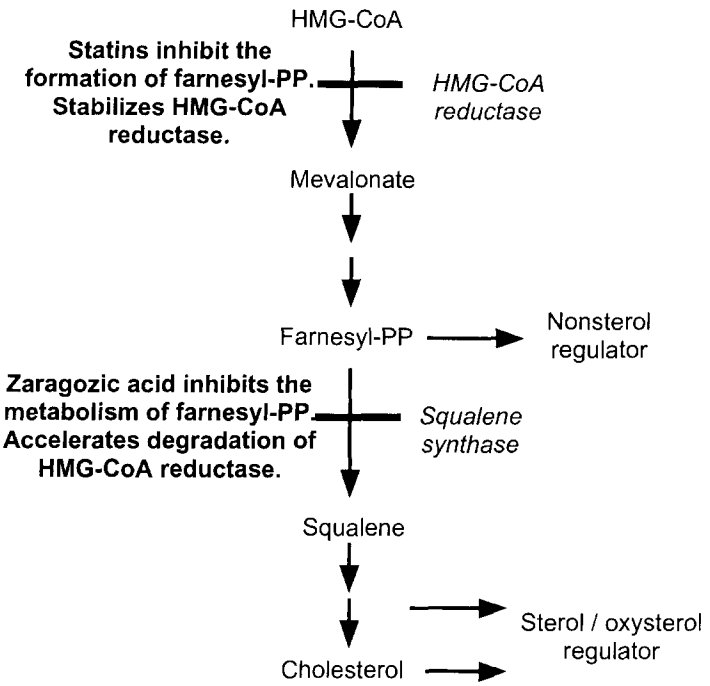

Fig. 7. Evidence that a farnesyl-PP-derived signal modulates HMG-CoA reductase degradation. In yeast, conditions that decrease farnesyl-PP levels stabilize HMG-CoA reductase levels whereas conditions that increase farnesyl-PP levels accelerate HMH-CoA reductase degradation.

tase degradation is derived from farnesyl-PP. This evidence has been recently reviewed (S.F. Petras, 2001) and includes the following. Intracellular farnesol levels increase significantly after mevalonate addition to cells (T.E. Meigs, 1996). Accelerated HMG-CoA reductase degradation can be induced in cells incubated with mevalonate, farnesyl-PP, or farnesol but not with a non-hydrolyzable analog of farnesyl-PP (C.C. Correll, 1994; M.D. Giron, 1994; T.E. Meigs, 1997). Furthermore, inhibition of the enzyme farnesyl pyrophosphatase blocks the mevalonate-dependent, sterol-accelerated degradation of the enzyme (T.E. Meigs, 1997).

Many, but not all, aspects of regulated HMG-CoA reductase degradation are conserved among eukaryotes. In yeast, modulation of Hmg2p stability by a farnesyl-PP-derived signal has been shown using pharmacologic and genetic approaches (R.G. Gardner, 1999). Conditions chosen to increase farnesyl-PP levels (inhibition of squalene synthase by zaragozic acid or down-regulation of squalene synthase) accelerated Hmg2p ubiquitination and degradation, whereas conditions chosen to decrease farnesyl-PP levels (inhibition of HMG-CoA reductase by statins, farnesyl-PP synthase down-regulation, or squalene synthase overexpression) stabilized Hmg2p (Fig. 7). One difference between the yeast and mammalian systems is the requirement for ongoing protein synthesis, which has not been shown in yeast (R.G. Gardner, 1999).

As mentioned above, regulation of mammalian HMG-CoA reductase turnover re-

quires both sterols and non-sterol isoprenoids. In contrast, the yeast isozyme can be suppressed by farnesyl-PP alone, although sterols enhance the non-sterol regulation. Pharmacologic and genetic approaches have again been extremely informative, showing that endogenously produced oxysterols serve as a positive signal for Hmg2P degradation in yeast (R.G. Gardner, 2001). Oxysterols have long been known to accelerate HMG-CoA reductase degradation when added to the medium of cultured mammalian cells; however, an endogenously produced oxysterol regulator of mammalian HMG-CoA reductase degradation has not yet been identified.

How do sterol or non-sterol regulators influence HMG-CoA reductase degradation? They could have a direct effect on the enzyme itself, as an allosteric regulator or by binding to the membrane domain. Alternatively, they could have an effect on the biophysical properties of the endoplasmic reticulum membrane (i.e. fluidity, membrane organization) or affect the proteolytic machinery. These possible effects could be direct or through interaction with an effector protein (R.G. Gardner, 1999; S.F. Petras, 2001).

The half-life of HMG-CoA reductase is also influenced by the enzyme's oligomerization state and expression level (H.H. Cheng, 1999). Oligomerization of HMG-CoA reductase through its cytosolic domain appears to stabilize the protein, as does a higher expression level. Analysis of the crystal structure of the catalytic portion of human HMG-CoA reductase revealed that this domain forms tight tetramers (E.S. Istvan, 2000). It was suggested that oligomerization of the catalytic domain may induce association of the membrane domains, which would decrease accessibility of the enzyme to proteases. Sterols may induce dissociation of the HMG-CoA reductase tetramer, resulting in accelerated proteolysis.

3.5. Sterol-sensing domain

How do HMG-CoA reductase and SCAP detect rising cellular cholesterol levels? A key feature of these proteins was revealed when SCAP was cloned and the deduced protein sequence compared with that of HMG-CoA reductase (X. Hua, 1996). A five transmembrane domain was found in SCAP with 25% identity and 55% similarity with a corresponding region in HMG-CoA reductase. This domain, termed the sterol-sensing domain, appeared to be critical for SCAP's cholesterol-regulated SREBP escort function since certain amino acid substitutions in the sterol-sensing domain led to constitutive activity (X. Hua, 1996; A. Nohturfft, 1996). In HMG-CoA reductase, transmembrane span 2 (which is within the sterol-sensing domain) was shown to be necessary for regulated degradation (H. Kumagai, 1995). Chimeric mutants of HMG-CoA reductase were constructed that combined membrane-spanning domains from the hamster enzyme (which is subject to regulated degradation) with membrane-spanning domains from the sea urchin enzyme (which shares 62% amino acid sequence identity with the hamster enzyme in the membrane domain, but is not subject to regulated degradation). Analysis of regulated turnover of the chimeric molecules showed that hamster transmembrane span 2 was sufficient to confer regulated degradation upon the sea urchin enzyme (H. Kumagai, 1995).

Sterol-sensing domains with a high degree of sequence similarity are found in several other proteins with obvious connections to cholesterol homeostasis. One is the

biosynthetic enzyme 7-dehydrocholesterol Δ7-reductase. The function of the sterol-sensing domain in 7-dehydrocholesterol Δ7-reductase is not clear; however, amino acid substitutions in that region cause the loss of 90% of catalytic activity (S.H. Bae, 1999). Another sterol-sensing domain-containing protein is NPC1, a 1278-amino-acid glycoprotein found in late endosomes and lysosomes (E.D. Carstea, 1997). NPC1 is hypothesized to play a role in trafficking of cholesterol, gangliosides and other cargo from late endosomes to destinations throughout the cell [24] (L. Liscum, 2000). Mutations in NPC1 lead to the predominant form of Niemann–Pick C disease, a human genetic disease characterized by progressive neurodegeneration. The biological function of NPC1 is still not clear, but structure/function analysis indicates that the sterol-sensing domain is important. Mutations that cause amino acid substitutions within the sterol-sensing domain lead to a rapidly progressing, infantile form of the disease, whereas amino acid substitutions throughout the rest of the protein cause the classical juvenile presentation [24]. NPC1L1 is a Golgi protein with 42% identity and 51% similarity with NPC1 (J.P. Davies, 2000). NPC1L1 has a sterol-sensing domain, but the function of this NPC1-like protein is unknown.

Other proteins with a sterol-sensing domain have a more tenuous link to cholesterol homeostasis. Two proteins, Patched and dispatched, are involved in developmental patterning (A.P. McMahon, 2000). Patched is the receptor for the morphogen, Sonic Hedgehog, which is the only known protein with a covalently attached cholesterol moiety. Dispatched is the plasma membrane protein required for secretion of cholesterol-modified Hedgehog. Hedgehog binding to Patched leads to a signal transduction cascade that activates transcription of specific genes. Mutations in Patched cause basal cell nevus syndrome, which is characterized by developmental abnormalities and basal cell carcinomas (R.L. Johnson, 1996). A role for Patched and dispatched in cholesterol metabolism has not been established; however, it is intriguing that exposure of embryos to inhibitors of cholesterol biosynthesis, such as Triparanol, AY-9944 or BM 15.766, cause profound developmental defects that resemble those in Sonic Hedgehog mutant embryos (J.A. Porter, 1996).

4. Metabolism of cholesterol

Cellular cholesterol levels are regulated, not only by feedback inhibition of cholesterol synthesis, but also by feedforward regulation of cholesterol metabolism. Excess cholesterol is metabolized to oxysterols. In addition to blocking SCAP-facilitated proteolysis of SREBP and thereby down-regulating endogenous cholesterol synthesis and LDL receptor levels, oxysterols also activate bile acid synthesis (discussed in Chapter 16) and cholesterol esterification, which further reduces the cellular content of unesterified cholesterol.

4.1. Oxysterols

Oxysterols are potent suppressors of cholesterol synthesis (A.A. Kandutsch, 1973, 1974). Their effectiveness has been attributed to their ability to diffuse into and through

cells to activate regulatory processes, thus bypassing the need for receptor-mediated entry. It was long assumed that cholesterol was the natural regulator and that oxysterols were contaminants found in commercial supplies of cholesterol or formed upon storage of stock cholesterol solutions. Now it is known that there are many naturally occurring oxysterols that have diverse actions on cellular lipid metabolism (G.J. Schroepfer, Jr., 2000). 25-Hydroxycholesterol is the most studied oxysterol; however, other oxysterols are as, or more, physiologically important.

Oxysterols can be formed by the action of at least three distinct hydroxylases [25]. The mitochondrial sterol 27-hydroxylase participates in an alternative pathway of bile acid biosynthesis, hydroxylating cholesterol and several other intermediates in the bile acid synthetic pathway. 27-Hydroxycholesterol formed in peripheral tissues is a potent inhibitor of endogenous cholesterol synthesis. It is also thought to be secreted into the bloodstream and transported to the liver, where 27-hydroxycholesterol binds the liver X receptor (LXR) nuclear hormone receptor (described in Chapter 16). LXR forms a heterodimer with the retinoid X receptor (RXR) and activates transcription of genes encoding bile acid biosynthetic enzymes. The physiological significance of sterol 27-hydroxylase is illustrated by the genetic disease cerebrotendinous xanthomatosis, which is caused by mutations in the sterol 27-hydroxylase gene [26]. The absence of this critical hydroxylase activity precludes the mobilization of excess cholesterol from peripheral tissues and leads to cholesterol deposition and xanthoma development.

24-Hydroxylase is an endoplasmic reticulum enzyme predominantly expressed in brain. Bjorkhem and colleagues have provided strong evidence that 24-hydroxylase maintains cholesterol homeostasis in the brain, which cannot participate in high-density lipoprotein-mediated reverse cholesterol transport (I. Bjorkhem, 1999). 24-Hydroxycholesterol is secreted from the brain into the circulation, taken up by the liver and metabolized into bile acids.

25-Hydroxylase is an endoplasmic reticulum and Golgi enzyme with low-level expression in most tissues [25]. 25-Hydroxycholesterol is a potent regulator of SREBP proteolytic processing. Given that the enzyme resides in the same subcellular compartment as SREBP and SCAP, 25-hydroxycholesterol may be a physiological regulator of cholesterol synthesis.

Oxysterol binding to LXR activates transcription of several genes that play key roles in maintaining bodily cholesterol homeostasis. One is the gene encoding the ATP-binding cassette transporter ABCA1, a 2201-amino-acid plasma membrane protein that stimulates cholesterol and phospholipid efflux. Cholesterol effluxed from peripheral cells by the action of ABCA1 is transferred by plasma high-density lipoproteins to the liver in a process called reverse cholesterol transport [27] (discussed in Chapter 20). A second example is LXR-activated transcription of the gene encoding cholesteryl ester transfer protein, which promotes transfer of cholesteryl esters from high-density lipoproteins to very low-density lipoproteins for clearance by the liver. Finally, oxysterol binding to LXR also stimulates expression of SREBP-1c, but not SREBP-1a or SREBP-2 (J.J. Repa, 2000). Therefore, increased cellular cholesterol should lead to oxysterol formation, which would increase expression of SREBP-1c and increase fatty acid synthesis. Indeed, administration of an LXR selective agonist to mice led to increased lipogenesis and higher plasma triacylglycerol and phospholipid levels (J.R. Schultz, 2000).

Another protein that binds oxysterols with high affinity was first reported by A.A. Kandutsch et al. (1977) and called oxysterol-binding protein (OSBP). At the time the protein was purified and cDNA-cloned (F.R. Taylor, 1989; P.A. Dawson, 1989), OSBP was expected to be a cytosolic protein that translocated into the nucleus and repressed transcription of cholesterogenic genes when oxysterols were present. Given our current knowledge of transcriptional control, we might expect OSBP to bind to the SREBP precursor or SCAP in the endoplasmic reticulum or site-1 protease or site-2 protease in the Golgi to interfere with SREBP proteolytic processing. However, a direct role for OSBP in transcriptional control has not been demonstrated. OSBP is a high-affinity 25-hydroxycholesterol-binding protein (K_d 10 nM) that translocates from cytosol and vesicles to the Golgi when ligand is bound [28].

How might OSBP transduce signals? One reasonable hypothesis is that when cellular cholesterol levels are high, cholesterol hydroxylation occurs. The resultant oxysterol then binds to OSBP, which translocates to the Golgi and signals suppression of cholesterol synthesis. However, OSBP responds paradoxically to cholesterol rather than oxysterols [28]. When cells are cholesterol replete, OSBP moves to the cytosol and vesicles, not to the Golgi. OSBP moves to the Golgi when cells are cholesterol-depleted. Thus, it has been difficult to establish the identity of OSBP's endogenous ligand and which downstream events are mediated by OSBP. The story is made more complex by the finding that the human OSBP family has at least five members, with sequence similarity in the C-terminal ligand-binding domain, whereas *Saccharomyces cerevisiae* has six related proteins [28].

4.2. Cholesteryl ester synthesis

Excess cholesterol can also be metabolized to cholesteryl esters. ACAT is the endoplasmic reticulum enzyme that catalyzes the esterification of cellular sterols with fatty acids. In vivo, ACAT plays an important physiological role in intestinal absorption of dietary cholesterol, in intestinal and hepatic lipoprotein assembly, in transformation of macrophages into cholesteryl ester laden foam cells, and in control of the cellular free cholesterol pool that serves as substrate for bile acid and steroid hormone formation. ACAT is an allosteric enzyme, thought to be regulated by an endoplasmic reticulum cholesterol pool that is in equilibrium with the pool that regulates cholesterol biosynthesis. ACAT is activated more effectively by oxysterols than by cholesterol itself, likely due to differences in their solubility. As the fatty acyl donor, ACAT prefers endogenously synthesized, monounsaturated fatty acyl-CoA.

The cloning of the human ACAT gene and its orthologs, as well as the subsequent generation of ACAT-deficient mice, led to the realization that two ACAT isozymes must contribute to the enzyme activity (reviewed in Farese [29], Rudel et al. [30] and Chang et al. [31]). Human ACAT-1 was cloned using an expression cloning strategy (C.C.Y. Chang, 1993). The gene encodes an integral membrane protein of 550 amino acids that is present in almost all cells and tissues examined. Orthologs were identified in other mammalian species, as well as *Drosophila melanogaster* and *Caenorrhabditis elegans*. The first indication of multiple ACATs came from the cloning of two ACAT-related enzymes (ARE1 and ARE2) from *S. cerevisiae* (H. Yang, 1996). The inactivation of both

yeast genes was required to eliminate sterol esterification. In addition, ACAT-1-deficient mice showed the expected depletion of cholesteryl esters in adrenals, ovaries, testes and macrophages, but no changes in intestinal cholesterol absorption or hepatic cholesterol esterification (V.L. Meiner, 1996). This result indicated that a second ACAT must be present in those mouse tissues.

The cloning of ACAT-2 (R.A. Anderson, 1998; S. Cases, 1998; P. Oelkers, 1998) revealed a protein of similar size to ACAT-1, with a novel N-terminus but a C-terminus highly similar to ACAT-1. In adult humans, ACAT-2 is confined to the apical region of intestinal enterocytes, with low levels also expressed in hepatocytes. Disruption of the ACAT-2 gene in mice led to dramatic reduction in cholesterol absorption and prevention of hypercholesterolemia (A.K.K. Buhman, 2000). The data suggest that, in humans, ACAT-1 plays a critical role in foam-cell formation and cholesterol homeostasis in extrahepatic tissues, whereas ACAT-2 has an important role in absorption of dietary cholesterol [31]. ACAT-1 is the major isozyme in hepatocytes, although the total pool of cholesteryl esters produced by both enzymes regulates very low-density lipoprotein synthesis and assembly [31].

5. *Future directions*

Fifty years ago, it was recognized that hepatic cholesterol synthesis was subject to feedback regulation by dietary cholesterol (R.G. Gould, 1950). Only in the last decade have the mechanisms been elucidated for transcriptional and degradative regulation of the rate-limiting enzyme, HMG-CoA reductase. Both forms of regulation require that proteins sense the local cholesterol concentration. Rising cholesterol levels cause HMG-CoA reductase to be ubiquitinated and degraded by the proteosome. They cause SCAP to remain localized to the endoplasmic reticulum rather than translocating to the Golgi. The challenge ahead is to determine how HMG-CoA reductase and SCAP transduce the signal of increased cellular cholesterol content into action, i.e. protein degradation or movement to Golgi.

HMG-CoA reductase and SCAP are not the only cellular proteins equipped with a sterol-sensing domain. Does the sterol-sensing domain in 7-dehydrocholesterol $\Delta 7$-reductase confer cholesterol-mediated feedback regulation upon this last step in the biosynthetic pathway? What is the function of the sterol-sensing domain in NPC1, NPC1L1, Patched and dispatched? Is their subcellular location or their binding to another protein altered by cholesterol? Finally, is sterol-sensing domain-mediated regulation due to the action of cholesterol itself or another biologically active sterol?

Abbreviations

ACAT	acyl-CoA : cholesterol acyltransferase
bHLH-Zip	basic helix–loop–helix leucine zipper
CE	cholesteryl ester
DHC	dehydrocholesterol

ER	endoplasmic reticulum
HMG	3-hydroxy-3-methylglutaryl
LDL	low-density lipoprotein
LXR	liver X receptor
OSBP	oxysterol-binding protein
PP	diphosphate
RXR	retinoid X receptor
S1P	site-1 protease
S2P	site-2 protease
SCAP	SREBP cleavage-activating protein
SLOS	Smith–Lemli–Opitz syndrome
SRE	sterol regulatory element
SREBP	SRE-binding protein

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