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The SREBP Pathway: Regulation of Cholesterol Metabolism by Proteolysis of a Membrane-Bound Transcription Factor

Review

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Animal cells must regulate their biosynthetic pathways so as to produce the required amounts of end-products without risking overproduction. Such control is particularly important in cholesterol homeostasis because cholesterol must be supplied for many cellular functions, including two recently recognized ones: formation of caveolae (Smart et al., 1994; Murata et al., 1995) and covalent modification of embryonic signaling proteins (Porter et al., 1996). Excess cholesterol must be avoided because it forms solid crystals that kill cells. Excess cholesterol in the bloodstream is also lethal because it deposits in arteries, initiating atherosclerosis (Small and Shipley, 1974). End-product regulation of cholesterol metabolism is achieved predominantly through repression of transcription of genes that govern the synthesis of cholesterol and its receptor-mediated uptake from plasma lipoproteins (Goldstein and Brown, 1990).

As an end-product repressor, cholesterol presents a special problem because it is an insoluble lipid that resides almost exclusively in cell membranes. How does the cell sense the level of a membrane-embedded lipid, and how is that information transmitted to the nucleus to regulate transcription? Answers are emerging from studies of a novel family of membrane-bound transcription factors called sterol regulatory element binding proteins (SREBPs) that regulate multiple genes involved in cholesterol biosynthesis and uptake. Here we review the SREBPs, focusing on the novel way in which sterols regulate their proteolytic release from membranes. Remarkably, insight into this processing may teach us about Alzheimer's disease, the most common degenerative disease of the brain, as well as coronary artery disease, the most common degenerative disease of the heart. Other aspects of SREBP physiology, such as the DNA binding activities and interactions with other transcription factors, have been discussed elsewhere (Kim et al., 1995; Osborne, 1995; Ericsson et al., 1996a; Magana and Osborne, 1996; Oliner et al., 1996).

From a historical perspective, the cholesterol biosynthetic pathway was the first anabolic pathway recognized to undergo end-product feedback suppression. More than 60 years ago, the balance studies of Schoenheimer and Breusch (1933) demonstrated that mice synthesize less cholesterol after they have ingested it in the diet. In the early 1950s, Gould et al. (1953) incubated liver slices from dogs and rabbits with [^{14}C]acetate and observed that its incorporation into cholesterol was reduced to less than 2% of the control value when cholesterol had been supplied in the diet. In the 1960s an important enzymatic site for this regulation was identified as the endoplasmic reticulum (ER) enzyme 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG CoA reductase), which converts HMG CoA to the 6-carbon intermediate, mevalonate (Bucher et al. 1960; Siperstein and Fagan, 1966). The latter is converted to isopentenyl pyrophosphate, which is polymerized and modified to form the 27 carbons of cholesterol. Cholesterol accumulation lowers the activity of HMG CoA reductase and several other enzymes in the cholesterol biosynthetic pathway, thereby limiting the production of cholesterol (reviewed in Goldstein and Brown, 1990).

The importance of the cholesterol feedback system to human health was established by the finding that diets rich in cholesterol and saturated fatty acids raise blood cholesterol levels and cause heart attacks (Keys, 1975). In addition to suppressing synthesis of cholesterol, high cholesterol diets act through the feedback system to reduce the liver's uptake of cholesterol by suppressing production of receptors for low density lipoproteins (LDL), thus causing these atherogenic particles to accumulate in blood (Spady et al., 1993; Goldstein et al., 1995). Conversely, the most potent cholesterol-lowering drugs, the HMG CoA reductase inhibitors, exploit the feedback system by blocking cholesterol synthesis, thereby reducing the liver's content of cholesterol and increasing its production of LDL receptors (Brown and Goldstein, 1986; Goldstein et al., 1995).

The SREBPs, which regulate transcription of HMG CoA reductase, also regulate transcription of genes encoding many other enzymes in the cholesterol biosynthetic pathway, including HMG CoA synthase, farnesyl diphosphate synthase, and squalene synthase (Goldstein and Brown, 1990; Osborne, 1995; Guan et al., 1995; Ericsson et al., 1996b). The SREBPs also regulate the LDL receptor, which supplies cholesterol through receptor-mediated endocytosis. In a surprising turn of events, the SREBPs, were found to modulate transcription of genes encoding enzymes of fatty acid synthesis and uptake, including acetyl CoA carboxylase, fatty acid synthase, stearoyl CoA desaturase-1, and lipoprotein lipase (Tontonoz et al., 1993; Kim and Spiegelman, 1996; Lopez et al., 1996; Magana and Osborne, 1996; Shimano et al., 1996). Thus, SREBPs coordinate the synthesis of the two major building blocks of membranes, fatty acids, and cholesterol.

SREBPs: Membrane-Bound Transcription Factors

The SREBPs were purified in 1993 from nuclear extracts of cultured human HeLa cells (Briggs et al., 1993; Wang et al., 1993), and their unique mechanism of regulation was also worked out in tissue culture (Wang et al., 1994). Three members of the SREBP family have been identified by cDNA cloning (Yokoyama et al., 1993; Hua et al., 1993). Each SREBP shares a similar tripartite structure, consisting of: (1) an NH_2-terminal transcription factor domain of ~480 amino acids; (2) a middle hydrophobic region of ~80 amino acids containing two hydrophobic transmembrane segments; and 3) a COOH-terminal regulatory domain of ~590 amino acids (Figure 1).

In humans, hamsters, and mice, two members of the SREBP family, designated SREBP-1a and SREBP-1c,

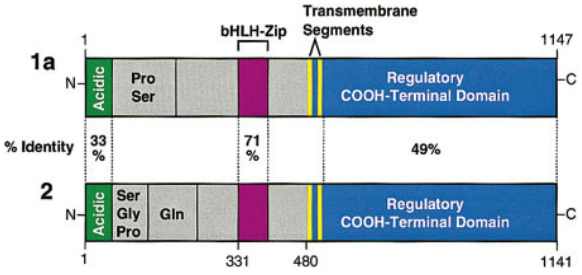

Figure 1. Domain Structures of Human SREBP-1a and SREBP-2

The sequence of SREBP-1c (not shown) is identical to that of SREBP-1a except for a shortened NH_2-terminal acidic domain (24 amino acids in SREBP-1c versus 42 amino acids in SREBP-1a).

are produced from a single gene (human chromosomal location, 17p11.2) through the use of alternate transcription start sites encoding alternate first exons that are spliced into a common second exon (Yokoyama et al., 1993; Hua et al., 1995b; Shimomura et al., 1997). The ADD1 transcription factor cloned from a rat adipocyte cDNA library by Tontonoz et al. (1993) is equivalent to SREBP-1c (Shimomura et al., 1997). The mRNAs for human SREBP-1a and -1c can also 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). This 3′ alternative splicing does not occur in the mouse (Hua et al., 1995b; Shimomura et al., 1997), and its functional significance is not apparent.

The third member of the SREBP family, designated SREBP-2, is encoded by a separate gene on human chromosome 22q13 (Hua et al., 1993; Miserez et al., 1997). Thus far, only one transcript from this gene has been demonstrated in humans, hamsters, and mice. Despite the differences in lengths of the human *SREBP-1* and *-2* genes (26 kb versus 72 kb), the locations of the exon–intron boundaries in both genes are similar (Miserez et al., 1997). A Drosophila homolog of the SREBPs has recently been cloned (Theopold et al., 1996). Its overall tripartite domain structure, including the two transmembrane segments, is virtually identical to that of the mammalian SREBPs.

The NH_2-terminal domain of ~480 amino acids of each SREBP is a transcription factor of the basic-helix-loop-helix-leucine zipper (bHLH-Zip) family. This segment begins with an acidic domain that resembles the ''acid blobs'' of many transcriptional activators (Figure 1). When this segment is deleted in transfection studies, the proteins retain the ability to bind to the relevant DNA sequence, but they lose their ability to activate transcription, so they are converted to inhibitors of transcription (Sato et al., 1994). The acidic NH_2-terminal domain in human SREBP-1a comprises 42 amino acids, of which 12 are acidic. In human SREBP-2, this domain contains 48 amino acids (14 acidic), but in SREBP-1c it contains only 24 amino acids (6 acidic). The shortened acidic domain in SREBP-1c, which results from use of a first exon containing a shorter coding region, renders SREBP-1c a much weaker activator of transcription than is SREBP-1a (Shimano et al., 1997) or SREBP-2. The acidic NH_2-terminal sequence is followed by sequences rich in proline, serine, glycine, and glutamine that vary between the SREBP-1s and SREBP-2. These sequences are followed by a classic bHLH-Zip sequence that resembles the sequence first identified in E12/E47, which binds to the immunoglobulin enhancer. Similar sequences are found in MyoD, Myc, and Max proteins, and in more than a score of other DNA binding proteins, all of which regulate transcription (Murre and Baltimore, 1992).

The basic regions of bHLH-Zip proteins bind to specific sequences in DNA, and the adjacent HLH-Zip regions mediate homo- or heterodimerization. All bHLH-Zip proteins other than SREBPs bind to target sequences called E boxes, which are inverted repeats of the sequence CANNTG (Murre and Baltimore, 1992). Although SREBPs can recognize E boxes in vitro, in the nucleus they bind to sequences that contain a direct repeat of 5′-PyCAPy-3′ (Magana and Osborne, 1996). In the LDL receptor the recognition site is 5′-ATCACCCAC-3′, which is designated sterol regulatory element 1 (SRE-1) (Yokoyama et al., 1993). Kim et al. (1995) showed that the ability of SREBPs to recognize a direct, rather than inverted, repeat depends on a tyrosine that replaces an arginine found in all other bHLH-Zip proteins. In their elegant studies of the crystal structure of the DNA binding and dimerization domains of SREBP-1a bound to SRE-1, Burley and coworkers (Parraga et al., unpublished data) explained the effects of the tyrosine substitution by showing that it enhances the mobility of the DNA binding domain, removing the requirement for an inverted repeat.

The SREBPs are distinguished from other members of the bHLH-Zip family by virtue of the unique sequence that follows the bHLH-Zip region, which consists of the following: (1) a hydrophobic membrane-spanning sequence of ~22 amino acids, (2) a hydrophilic ''lumenal loop'' of ~31 amino acids, (3) a second hydrophobic membrane-spanning sequence of ~22 amino acids, and (4) a long COOH-terminal extension of ~590 amino acids that is necessary for sterol-regulated cleavage (Figure 1). Immunofluorescence, protease protection, and glycosylation site tagging experiments indicate that the SREBPs are bound to membranes of the ER and nuclear envelope in a hairpin fashion with the large NH_2-terminal and COOH-terminal segments facing the cytoplasm and the short 31-residue lumenal loop projecting into the lumen of the organelle (Hua et al., 1995a; Duncan et al., 1997).

A Proteolytic Cascade

In order to influence transcription, the NH_2-terminal domain of SREBPs must be released from the membrane so that it can enter the nucleus. This release has been studied most extensively for SREBP-2. Release is accomplished by a two-step proteolytic cascade that is regulated by sterols (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 SREBP-2 at site 1, which is located approximately in the middle of the lumenal loop (Figure 2, upper panel). The site 1 protease cleaves between the leucine and serine in the sequence RSVL S (Duncan et al., 1997). Cleavage is absolutely dependent on a positively charged residue, either arginine or lysine, at the P4 position. The serine and valine

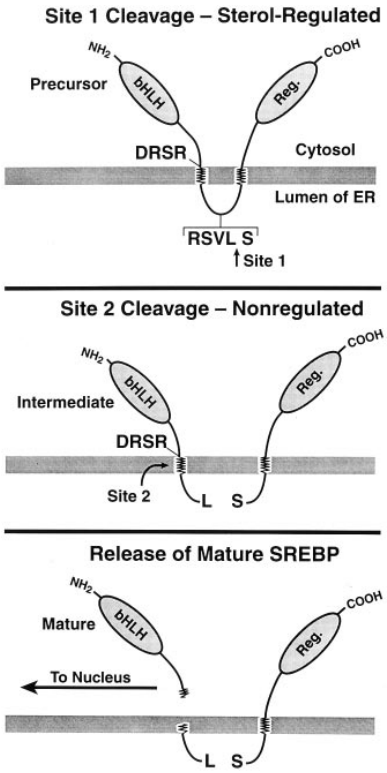

Figure 2. Model for Two-Site Proteolytic Cleavage of Membrane-Bound SREBPs

Abbreviations: bHLH, basic-helix-loop-helix-leucine zipper domain of SREBP; Reg., regulatory domain of SREBP.

at the P3 and P2 positions can be replaced with alanines without loss of cleavage. Leucine is strongly preferred at the P1 position. Substitutions with valine, alanine, phenylalanine, arginine, or glutamic acid either abolish or markedly reduce cleavage activity. The serine at the P′1 position can be replaced with a variety of amino acids without affecting cleavage. Although single alanine substitutions at other positions in the lumenal loop do not reduce cleavage, it is believed that structural features of the surrounding sequences are required since movement of the RSVL tetrapeptide to other positions in the lumenal loop markedly decreases cleavage (Duncan et al., 1997).

Cleavage at site 1 breaks the covalent bond between the two transmembrane domains of SREBP-2, but both halves of the protein remain attached to membranes (Figure 2, middle panel). Shortly thereafter, a second protease clips the NH_2-terminal fragment at site 2, which appears to reside within the first transmembrane domain (Sakai et al., 1996). This releases the mature NH_2-terminal domain into the cytosol, from which it rapidly enters the nucleus, apparently with a portion of the transmembrane domain still attached at the COOH terminus (Figure 2, lower panel). Cleavage at site 2 is strongly dependent on the tetrapeptide DRSR that precedes the first transmembrane domain (Hua et al., 1996b; Sakai et al., 1996). Substitutions in this tetrapeptide abolish cleavage at site 2, and the NH_2-terminal fragment remains in a membrane-bound state.

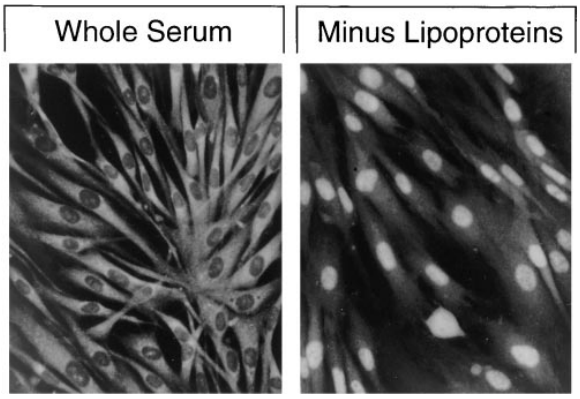

Figure 3. Immunofluorescence of SREBP-2 in Cultured Fibroblasts Grown in Whole Serum or Lipoprotein-Deficient Serum

Monolayers of diploid human fibroblasts were cultured for 4 days in 10% fetal calf serum. On day 5, the cells were either re-fed with 10% fetal calf serum (left panel) or switched to 10% lipoprotein-deficient serum (right panel). On day 6, the cells were fixed with paraformaldehyde and stained with a rabbit anti-SREBP-2 polyclonal antibody (affinity purified), followed by fluorescein-labeled goat anti-rabbit IgG. Magnification, 450×.

Cleavage at site 1 is tightly regulated by sterols (Wang et al., 1994; Sakai et al., 1996). When cultured cells such as CHO cells, HeLa, or human embryonic kidney 293 cells are deprived of sterols, cleavage at site 1 is active. This cleavage is abolished when cells are overloaded with sterols by incubation with LDL or a mixture of cholesterol and 25-hydroxycholesterol. Cleavage at site 2 is not directly regulated by sterols, but it requires prior cleavage at site 1. Hence, it occurs only in sterol-depleted cells.

The sterol-regulated proteolysis of SREBPs is illustrated visually in Figure 3, which shows cultured human fibroblasts that were stained for endogenous SREBP-2 with an antibody directed against the NH_2 terminus. In fibroblasts grown in whole serum, which supplies LDL-cholesterol, SREBP-2 was located in extranuclear membranes (left panel). When the cells were deprived of cholesterol by growth in lipoprotein-deficient serum, the NH_2-terminal fragment of SREBP-2 was released from the membranes and entered the nucleus (right panel). SREBP-1 behaves similarly in these cells.

The crucial role of the proteolytic cascade in sterol regulation is revealed by transfection experiments with cDNAs encoding truncated versions of the SREBPs that terminate before the first transmembrane domain (Sato et al., 1994; Yang et al., 1994, 1995). These proteins contain the bHLH-Zip domain and all of the other sequences necessary for transcriptional activation; however, they are never attached to membranes, and they enter the nucleus directly without a requirement for proteolysis. In cells expressing these truncated proteins, the SREBP-dependent genes are continuously activated, and they cannot be suppressed by sterols. When these cells are grown in the presence of serum that contains LDL, they fill up with huge amounts of cholesteryl esters, owing to continuous nonregulated synthesis of cholesterol and uptake of LDL.

Thus far there is no in vitro biochemical description

of the proteases that cleave at site 1 or site 2. Both are predicted to be unusual enzymes. The site 1 protease is unusual in that it appears to function in the lumen of the ER, and it recognizes a tetrapeptide sequence, RSVL, which is unlike any known protease substrate. The site 2 protease is unusual because it appears to cut SREBP within a membrane-spanning segment as estimated from a careful analysis of the size of the liberated fragments (Sakai et al., 1996). The location of site 2 in the transmembrane sequence is consistent with sequence analysis of tryptic peptides prepared from SREBP-1 that was purified from nuclear extracts of HeLa cells (Yokoyama et al., 1993). One of the tryptic peptides (peptide 1) terminated at an arginine that is 4 residues to the NH_2-terminal site of the DRSR sequence. This confirms that the nuclear NH_2-terminal fragment of SREBP-1 extends at least to the first transmembrane domain.

The cellular locations of the two proteases are not known. Immunofluorescence studies show that the full-length SREBP precursors are located in the ER and nuclear envelope (Sato et al., 1994). Moreover, when N-linked glycosylation sites were inserted into the lumenal loop, the carbohydrate chains on the precursor remained in the high mannose endoglycosidase H–sensitive form, indicating that the precursor had not been transported to the Golgi complex (Hua et al., 1995a; Duncan et al., 1997). We therefore believe that the site 1 protease acts in the lumen of the ER and the contiguous nuclear envelope. However, we cannot exclude the possibility that prior to cleavage the precursor is transported to the Golgi complex where cleavage immediately occurs. The site 2 protease may also function in the ER, but it seems equally likely that this enzyme is located in a more distal site such as the Golgi complex. By one hypothetical scenario, cleavage at site 1 in the ER separates the two halves of the protein, allowing the NH_2-terminal intermediate to move to the Golgi where it encounters the site 2 protease.

SREBP Cleavage Activation Protein, SCAP

Recently an important regulator of SREBP processing, designated SCAP, was cloned from 25-RA cells, a mutant line of CHO cells that fails to suppress cleavage of SREBPs in the presence of sterols (Hua et al., 1996a). These cells continue to synthesize cholesterol and to take up LDL even when they are massively overloaded with sterols. Cell fusion studies by Hasan and Chang (1994) showed that the defect was semidominant, presumably representing a gain of function. Transient transfection of a cDNA library from 25-RA cells into 293 cells allowed our laboratory to isolate a cDNA that encodes a mutant version of a novel protein named SCAP (SREBP cleavage activating protein.) (Hua et al., 1996a). The mutant SCAP stimulates cleavage of SREBPs, even in the presence of sterols, thus explaining the sterol-resistant phenotype of 25-RA cells.

SCAP is a membrane-bound protein with two domains. The NH_2-terminal 730 amino acids consist of alternating hydrophobic and hydrophilic sequences. These are predicted to form up to eight membrane-spanning sequences separated by short hydrophilic loops (Hua et al., 1996a). The hydrophilic COOH-terminal domain of SCAP comprises 546 amino acids organized into four repeats of a sequence belonging to a family designated WD, which are reiterated in many proteins (Neer et al., 1994). The best understood is the β subunit of heterotrimeric G proteins, which contains seven WD repeats that are arrayed in a fan shape like the blades of a propeller (Wall et al., 1995; Sondek et al., 1996). Variable sequences on the surface of each blade interact with other proteins. WD repeats have been found in more than 30 proteins, and in each case they have been postulated to mediate protein–protein interactions (Neer et al., 1994).

The most intriguing aspect of SCAP is the sequence resemblance of its hydrophobic NH_2-terminal domain to the corresponding domain of HMG CoA reductase (Hua et al., 1996a). Like SCAP, HMG CoA reductase has an NH_2-terminal membrane-attachment domain with eight membrane-spanning segments (Olender and Simoni, 1992). It also has a cytoplasmic COOH-terminal domain that contains all of the catalytic activity (Liscum et al., 1985). The NH_2-terminal domain imparts sterol regulation. In sterol-depleted cells the enzyme has a long half-life, but it is degraded rapidly when sterols accumulate. Regulated degradation depends on the membrane attachment domain, and it is abolished when this domain is deleted (Gil et al., 1985). Studies with chimeric proteins have also implicated the membrane-spanning domain in the sterol-sensing function (Kumagai et al., 1995). The NH_2-terminal domain of SCAP (amino acids 280–444) shows 25% identity and 55% similarity to the region of HMG CoA reductase that corresponds to membrane-spanning segments 2–6, implying that this domain in SCAP, like its counterpart in HMG CoA reductase, has a sterol-sensing function (Hua et al., 1996a).

The mutant SCAP produced in 25-RA cells has an asparagine substituted for aspartic acid at residue 433, which is just at the cytoplasmic side of the last of the membrane-spanning segment that resembles HMG CoA reductase (Hua et al., 1996a). When expressed at low levels that approximate physiologic concentrations, the mutant SCAP stimulates cleavage of SREBPs at site 1, and it renders the cleavage reaction insensitive to inhibition by sterols. Wild-type SCAP can have a similar effect, but only when expressed at 10-fold higher levels. The mutant 25-RA cells have one copy of the mutant SCAP and one copy of the wild-type gene, and yet they exhibit a sterol-resistant phenotype, attesting to the gain of potency imparted by the D443N mutation (Hua et al., 1996a). Remarkably, the same nucleotide substitution (G→A in codon 443) recurred in two other CHO cell lines that were mutagenized and selected for sterol resistance. Although the mutagens used favor G→A transitions, the involvement of the same G in all three of these cell lines attests to the specificity of the D443N substitution in rendering SCAP sterol resistant (Nohturfft et al., 1996).

We interpret these data to indicate that SCAP is a required activator of SREBP cleavage and that the activity of the wild-type protein is abolished by sterols. Mutant SCAP is both superactive and sterol resistant. SCAP may act by binding SREBPs, perhaps through an interaction of the cytoplasmic WD repeat region of

Table 1. Mutant Hamster Cells with Defects in SREBP Processing

Phenotype	Molecular Defect	Mutant Cell Lines
Sterol-Resistant[a]		
Class 1	Truncated SREBP-2 at amino acid 460	SRD-1, SRD-2, SRD-3
Class 2	Activating mutation in SCAP, Asp 443→Asn	25-RA, SRD-4, SRD-8
Sterol-Requiring[b]	Failure to cleave SREBP-1 and SREBP-2 at Site 2	M19, SRD-6

[a] Unable to repress synthesis of cholesterol and LDL receptors in response to sterol excess.
[b] Unable to induce synthesis of cholesterol and LDL receptors in response to sterol depletion.

SCAP and the cytoplasmic COOH-terminal domain of SREBP. Although there are no published data to support this model, it is attractive for two reasons: (1) the COOH-terminal regulatory domain of SREBPs is required for site 1 cleavage (Hua et al., 1996b); and (2) WD repeats, in general, mediate protein–protein interactions. If SREBP–SCAP binding does occur, the complex might serve as the site of attachment of the lumenal protease that cuts SREBPs at site 1. SCAP itself has no hallmarks of a protease, and to date none of the proteins that contain WD repeats has been shown to be an enzyme. If SCAP–SREBP binding is necessary for site 1 proteolysis, it is possible that sterols disrupt proteolysis by blocking this binding. The simplest mechanism would invoke a sterol-mediated increase in the degradation of SCAP, much as sterols accelerate the degradation of HMG CoA reductase. So far, however, we have been unable to obtain evidence that sterols acutely decrease the amount of immunodetectable SCAP in cultured cells.

Mutant Cells with Defects in Processing of SREBPs

Two types of regulatory mutants in sterol metabolism have been isolated in appropriate selective media: (1) sterol-resistant cells, like the 25-RA cells described above, that fail to suppress enzymes of cholesterol synthesis or LDL receptors in response to cholesterol or oxygenated sterols such as 25-hydroxycholesterol; and (2) cholesterol auxotrophs, which fail to induce the enzymes of cholesterol synthesis or LDL receptors upon sterol deprivation (reviewed in Chang et al., 1997). Both of these phenotypes result from defects in regulation of SREBP cleavage. Table 1 reviews the mutant hamster cell lines in which the molecular defect has been characterized.

The sterol-resistant mutants are divided into two classes. Class 2 includes the 25-RA cells and other CHO cell lines that produce a dominantly acting mutant SCAP. Class 1 mutants are sterol resistant because they produce a nonsuppressible form of SREBP-2 that is truncated at residue 460, which is between the bHLH-Zip domain and the first transmembrane segment. Three mutant cell lines of this class have been isolated, and all have undergone recombinations in the intron following the exon that terminates at codon 460 of *SREBP-2* (Yang et al., 1994, 1995). The resulting mRNAs encode fusion proteins whose SREBP-2 sequence terminates at codon 460. Since these proteins are never attached to membranes, they proceed to the nucleus directly without requiring proteolysis. Thus, they are immune from sterol down-regulation, and they confer a sterol-resistant phenotype. It is interesting that each of the three mutants involves SREBP-2, and not SREBP-1, and that each recombination involves the intron following codon 460, and not the intron following codon 400, which should have produced a similar phenotype (Yang et al., 1995). The recurrent involvement of the same intron is not attributable to a recombination ''hot spot'' since different sites of recombination are found in each of the mutants. This recurrence more likely results from the size of the intron in *SREBP-2* ($\sim$3100 bp), which is larger than the other introns at which recombination could produce the same phenotype (Yang et al., 1995). Apparently, the large intron in *SREBP-2* increases the probability that a recombination event will occur. When this creates a desirable phenotype, such as escape from sterol-mediated down-regulation, the cells gain the ability to survive under the selective conditions employed. This finding illustrates the advantage of introns in providing the genomic diversity that allows rapid evolution by recombination and selection.

The other type of CHO cell mutant, the cholesterol auxotroph, has also taught us much about SREBP processing (Table 1). The original cell line with this phenotype, M19 cells, was isolated by Chin and Chang (1981). The cells were shown to have three phenotypic abnormalities: (1) failure to induce cholesterol biosynthetic enzymes such as HMG CoA synthase and HMG CoA reductase upon sterol deprivation, thus explaining the cholesterol auxotrophy; (2) failure to induce the LDL receptor, indicating that the cells have a global failure to induce sterol-repressed genes upon sterol deprivation; and (3) a requirement for unsaturated fatty acids, in addition to cholesterol, for cell growth. As discussed below, all of these defects can now be traced to a single abnormality: failure of the site 2 protease to release the NH_2-terminal fragment of SREBPs from cell membranes.

In recent studies, Sakai et al. (1996) showed that two independently derived cholesterol auxotrophic CHO cell lines, the M19 cells of Chin and Chang (1981) and the SRD-6 cells from our laboratory (Evans and Metherall, 1993), exhibited normal cleavage of SREBPs at site 1 following sterol deprivation. However, cleavage at site 2 did not follow, and the NH_2-terminal fragments of SREBPs accumulated in the membrane-bound intermediate form. Hasan et al. (1994) showed that the defect in M19 cells is recessive and that it can be corrected by transfection of DNA from a human genomic library. This finding should permit the cloning of the gene that is defective in the M19 cells. This gene may encode the site 2 protease or another protein whose action is required for cleavage by this enzyme.

The defect in site 2 proteolysis and the consequent failure of SREBPs to enter the nucleus explain the failure to produce cholesterol and LDL receptors. But do they

explain the requirement for unsaturated fatty acids? An answer to this question was suggested recently by studies in transgenic mice which indicate that SREBPs may be required to activate transcription of the gene encoding stearoyl CoA desaturase 1, an enzyme that synthesizes unsaturated fatty acids (see below).

SREBPs and Fatty Acid Synthesis

SREBPs were first implicated in regulating fatty acid synthesis by Tontonoz et al. (1993), who isolated a cDNA encoding rat ADD1 in a screen for adipose tissue cDNAs that encode bHLH proteins that bind to E boxes. ADD1 is the rat homolog of human and mouse SREBP-1c (Shimomura et al., 1997). Tontonoz et al. (1993) showed that overexpression of ADD1/SREBP-1c stimulated transcription of a reporter gene driven by a polymerized set of E boxes derived from the fatty acid synthase promoter. Magana and Osborne (1996) then showed directly that the NH_2-terminal domain of SREBP-1a and SREBP-2 activates the authentic fatty acid synthase promoter. Surprisingly, this effect was mediated not by the E box, but by an adjacent sequence that contains two SRE-1-like sequences that flank the E box. Lopez et al. (1996) showed that SREBP-1a stimulates transcription of the gene encoding acetyl CoA carboxylase, which provides the malonyl CoA substrate for fatty acid synthase. When transfected into cultured 3T3-L1 preadipocytes, ADD1/SREBP-1c enhances the program of adipocyte differentiation by stimulating expression of genes for fatty acid biosynthesis (Kim and Spiegelman, 1996).

The implication of SREBPs in regulating fatty acid synthesis was surprising since nuclear SREBPs are suppressed by cholesterol and since cholesterol overaccumulation was not known to suppress fatty acid biosynthesis. In the classic experiments of Gould et al. (1953) and Siperstein and Fagan (1966), the feeding of cholesterol to animals shut down cholesterol synthesis in the liver, but it had only small effects on fatty acid synthesis. Similarly, in the early experiments of Goldstein et al. (1974) in human fibroblasts, cholesterol overloading nearly abolished cholesterol synthesis from acetate, but it did not substantially reduce fatty acid biosynthesis. The lack of an absolute requirement for SREBPs in fatty acid biosynthesis is supported by observations in the mutant M19 line of CHO cells described above. These cells fail to process SREBPs normally and they therefore require exogenous cholesterol for growth. The rate of cholesterol synthesis in these cells is $<5\%$ of normal, indicating that this process absolutely requires SREBPs, whereas the rate of fatty acid synthesis is $\sim 30\%$ of normal (Pai et al., unpublished data).

These observations can be reconciled by the suggestion that SREBPs act as auxiliary regulators of the fatty acid biosynthetic enzymes by elevating their transcription above a basal level under certain circumstances. Under other circumstances, alternative factors can replace the SREBPs.

SREBPs in Liver

All of the early studies of SREBPs were performed in cultured cells, and the results have only recently been extended to in vivo metabolism. These studies focused primarily on the liver because of its importance in whole-body homeostasis of cholesterol and fatty acids. The results have highlighted important contrasts between liver and cultured cells.

In a large variety of cultured cells, including the HepG2 line of human hepatocytes and the 3T3-L1 line of mouse preadipocytes, expression of SREBP-1a, with its long acidic activation domain, exceeds that of SREBP-1c by more than a 2:1 ratio (Shimomura et al., 1997). SREBP-2 is also produced at high levels in these cells. The proteolytic processing of SREBP-1a and SREBP-2 is regulated in parallel. They are both cleaved at site 1 (and therefore at site 2) when cells are deprived of sterols, and their cleavage is suppressed by sterols.

A different situation exists in livers of hamsters. When hamsters were fed a typical low-fat chow diet, their liver nuclei contained the mature form of SREBP-1, but relatively little SREBP-2 (Sheng et al., 1995). When the liver was depleted of cholesterol by feeding a diet containing the cholesterol synthesis inhibitor lovastatin and the bile acid–binding resin Colestipol, the total amount of SREBP-2 increased, and the efficiency of its proteolytic processing rose markedly, as expected. Paradoxically, the processing of SREBP-1 became less efficient. As a result, sterol-depleted liver nuclei contained much more mature SREBP-2 than SREBP-1 (Sheng et al., 1995). This rise in SREBP-2 was accompanied by a marked increase in the amounts of target mRNAs for cholesterol-related genes, including HMG CoA reductase, HMG CoA synthase, and the LDL receptor. A reason for the reciprocal regulation of SREBP-1 and -2 in liver was suggested when the relative distribution of SREBP-1a and -1c were studied in this organ (Shimomura et al., 1997). In livers of mice and humans, the SREBP-1c transcript exceeded the -1a transcript by 9-fold, which is directly opposite to the ratio in cultured cells. If this distribution in hamster liver is similar to that in liver of mice and humans, then in liver cholesterol depletion specifically inhibits the processing of SREBP-1c at the same time that it activates the processing of SREBP-2.

The potential significance of these tissue-specific differences became apparent when the transcriptional activities of SREBP-1c and SREBP-1a were compared quantitatively by transfection into cultured cells. When expressed at levels that are many-fold above the physiologic range, the mature NH_2-terminal fragments of SREBP-1a and -1c were both able to stimulate transcription of reporter genes driven by the promoters for the LDL receptor, HMG CoA synthase, and fatty acid synthase (Shimano et al., 1997). However, at lower levels of expression that approximated the physiologic range, SREBP-1a was 10-fold more potent than SREBP-1c with respect to the LDL receptor and HMG CoA synthase promoters, but only about 2-fold more potent in stimulating fatty acid synthase (Shimano et al., 1997).

The liver is not the only organ that produces an abundance of SREBP-1c relative to -1a. A similar situation exists in mouse adipose tissue where the level of *SREBP-1c* mRNA exceeds that of *SREBP-1a* by 3-fold (Shimomura et al., 1997). Here the contrast between in vivo and in vitro is particularly striking since cultured preadipocytes (mouse 3T3-L1 cells) produce SREBP-1a almost exclusively, even after they have been induced

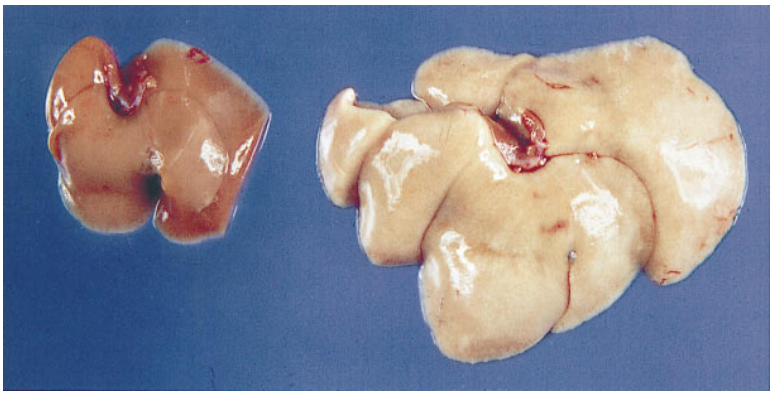

Figure 4. Livers from 18-Week-Old Littermate Wild-Type and Transgenic Mice

The transgene encodes a truncated version of SREBP-1a (amino acids 1–460) driven by the phosphoenolpyruvate carboxykinase promoter. Both animals (wild type, left; transgenic, right) were fed a low carbohydrate/high protein diet for 2 weeks to induce expression of the transgene.

to differentiate into adipocytes in tissue culture (Shimomura et al., 1997). This differentiation is known to be accompanied by a marked increase in *SREBP-1* mRNA (Kim and Spiegelman, 1996), but the mRNA that increases is *SREBP-1a*, not *SREBP-1c* (also known as *ADD1*).

As mentioned above, SREBP-1c retains some ability to stimulate fatty acid synthesis, but it has very little ability to stimulate cholesterol synthesis (Shimano et al., 1997). These results have led to a working model that postulates that liver and other adult organs produce SREBP-1c in the basal state to support fatty acid synthesis, albeit at a low level. When demands for cholesterol increase, the liver produces more SREBP-2 and processes it more efficiently, thereby activating cholesterol synthesis. Tissue culture cells, on the other hand, need cholesterol constantly for membrane synthesis during growth, and they produce SREBP-1a as well as SREBP-2, which gives them a higher potential for cholesterol synthesis.

The dramatic consequences of overproducing a dominant positive form of SREBP-1a in mouse liver are shown in Figure 4. In these experiments, Shimano et al. (1996) produced transgenic mice that overexpress an NH_2-terminal fragment of human SREBP-1a that terminates at residue 460, which is between the bHLH-Zip domain and the first transmembrane domain. Expression was driven by the phosphoenolpyruvate carboxykinase promoter, which gives high level expression in the liver and other organs when the animals are placed on a low carbohydrate/high protein diet. The livers of these animals were massively enlarged (up to 4-fold above normal), owing to massive engorgement with triglycerides and cholesteryl esters. Despite this engorgement, which should have triggered feedback repression, the livers continued to synthesize cholesterol and fatty acids at rates that were 5-fold and 20-fold greater than normal, respectively. The mRNAs for the cholesterol biosynthetic enzymes HMG CoA synthase, HMG CoA reductase, and squalene synthase were elevated from 5- to 37-fold. The mRNAs for the fatty acid–synthesizing enzymes, acetyl CoA carboxylase and fatty acid synthase, were elevated 17- to 20-fold, and the mRNA encoding stearoyl CoA desaturase 1 was increased by 4-fold. There was also a 6-fold increase in the mRNA for the LDL receptor and a 25-fold increase in the mRNA encoding lipoprotein lipase.

The nonregulated expression of SREBP-1a in liver nuclei triggered a program of lipid synthesis and sequestration, rather than one of lipid secretion. Despite the massive increase in stores of triglycerides and cholesteryl esters, there was no increase in the mRNAs for apolipoproteins required for lipoprotein assembly and secretion (such as apo B, apo E, and apo A-I) (Shimano et al., 1996). Plasma triglyceride levels were actually lower in the transgenics than in wild-type littermates fed the same diet (Shimano et al., 1997). There was also a progressive decrease in the mass of white adipose tissue throughout the body. It is not known whether the latter is attributable to the high-level expression of the SREBP-1a NH_2-terminal fragment in adipose tissue nuclei, or whether it is a consequence of fat accumulation in the liver, which might deprive adipose tissue of fatty acids through a ''triglyceride steal'' syndrome (Shimano et al., 1996).

In contrast to the truncated SREBP-1a, which had profound effects on hepatic lipid metabolism, overexpression of the corresponding version of SREBP-1c had a much less pronounced effect in transgenic mice (Shimano et al., 1997). The livers were mildly enlarged (~33% above normal), and there was a slight increase in cholesterol content and a more pronounced 4-fold increase in triglycerides. The levels of the mRNAs for the LDL receptor and cholesterol biosynthetic enzymes were not elevated, and the mRNAs for acetyl CoA carboxylase and fatty acid synthase were increased by only 2- to 4-fold as compared with 20-fold in the transgenics expressing comparable amounts of truncated SREBP-1a. These results reflect the relatively weak ability of SREBP-1c to stimulate transcription, as discussed above. They also point out that SREBP-1c seems to exert a greater effect on the fatty acid biosynthetic pathway as opposed to the cholesterol biosynthetic pathway.

Thus far, there are no published studies of transgenic mice expressing the NH_2-terminal domain of SREBP-2. Inasmuch as this protein is normally up-regulated in response to demands for cholesterol (Sheng et al., 1995), it seems likely that the SREBP-2 transgenics should show a relatively selective increase in cholesterol biosynthesis. Experiments to test this hypothesis are under way.

Parallels between Processing of SREBPs and β-Amyloid Precursor Protein

β-Amyloid precursor protein (βAPP) is a 770–amino acid cell surface protein with a single membrane-spanning

segment that is oriented with its NH_2 terminus on the extracytoplasmic face and its COOH terminus in the cytoplasm. It is produced in neurons and other cells. For unknown physiologic reasons, some βAPP molecules are processed by a series of proteolytic cuts to generate small peptides of 40–42 amino acids known as amyloid β-peptides. Because of their β-pleated sheet structure, these peptides aggregate to form insoluble amyloid deposits that lead to neural degeneration and eventually produce Alzheimer's disease (reviewed in Selkoe, 1996).

The proteolytic processing of βAPP is thought to occur during its transit from the ER to the cell surface (Selkoe, 1996; De Strooper et al., 1997). In many respects this process resembles the processing of SREBPs. First, the lumenal segment of βAPP is cut by a protease called β-secretase that clips the protein at a position 28 amino acids from the membrane. This protease is analogous to the SREBP site 1 protease, which cuts at a position 20 residues to the lumenal side of the first membrane-spanning segment. Next βAPP is processed by a second protease, called γ-secretase, that cuts within the membrane-spanning segment at one of two sites, thereby generating an amyloid β-peptide of 40–42 residues. This protease is analogous to the SREBP site 2 protease and is the only other protease that is suspected to cut a protein within a membrane-spanning segment. The released amyloid β-peptide is analogous to the short connecting segment (~25 amino acids) that is postulated to be released on the lumenal surface when the intermediate form of SREBP is cut by the site 2 protease (Figure 2, bottom panel).

βAPP processing is stimulated by several polytopic membrane proteins called presenilins (Lamb, 1997). Point mutations in presenilins increase the processing at one of the γ-cleavage sites to enhance production of the toxic 42-residue form of amyloid β-peptide (Citron et al., 1997). The presenilins have been localized to the ER and Golgi complex (De Strooper et al., 1997). Although presenilins have no sequence resemblance to SCAP, the proteins are alike in their polytopic membrane character, cellular localization, stimulatory action, and activation by point mutation. There are two differences between the processing of βAPP and the SREBPs. First, the membrane orientation is reversed. The NH_2-terminus of SREBPs faces the cytoplasm, whereas the opposite is true for βAPP. Second, the presenilins are thought to act primarily by stimulating the γ-secretase (analogous to site 2 in SREBPs), whereas SCAP stimulates cleavage at site 1. We cannot rule out the possibility that SCAP stimulates cleavage at both sites, and the same may be true of the presenilins. Despite these two differences, the parallels between the processing of SREBPs and βAPP suggest that one or both proteases may be members of the same family.

Further progress in understanding SREBP processing will require identification and molecular characterization of the proteases that cleave at site 1 and site 2. When these enzymes are identified, they may provide clues to the understanding of amyloid-induced degenerative brain disease as well as cholesterol-induced coronary heart disease.

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