Cellular and Molecular Characterization of the Sterol-Regulatory Element-Binding Protein-1

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to my parents

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Abbreviations

aa	amino acid(s)
ADD -1	adipocyte differentiation and determination factor one
ANOVA	analysis of variance
ART	antiretroviral treatment
ATP	adenosine triphosphate
bp	base pairs
bHLH-Zip	basic helix-loop-helix-leucine zipper
BSA	bovine serum albumin
cDNA	complementary deoxyribonucleic acid
CHO	chinese hamster ovary
CMV	cytomegalovirus
CoA	coenzyme A
Ct	threshold cycle value
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	deoxynucleotide triphosphate
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmic reticulum
ESE	exonic splicing enhancers
FAM	6-carboxy-fluorescein
FAS	fatty acid synthase
FBS	fetal bovine serum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
h	hour(s)
HEK	human embryonic kidney
HeLa	human cervix carcinoma
HepG2	human hepatocellular carcinoma
HIV	human immunodeficiency virus
HMG-CoA	S-3-hydroxy-2-methylglutaryl-coenzyme A
kb	kilo base

kDa	kilo Dalton
LDL	low density lipoprotein
LDLR	low density lipoprotein receptor
LPDS	lipoprotein-deficient serum
LPL	lipoprotein lipase
MGB	minor groove binder/non-fluorescent quencher
mRNA	messenger ribonucleic acid
NADPH	nicotinamide adenine dinucleotide phosphate
NMD	nonsense mediated mRNA decay
nt	nucleotide(s)
ORF	open reading frame
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PI	protease inhibitor
PPAR-γ	peroxisome proliferator-activated receptor-gamma
PTC	premature translation termination codon
RLU	relative light units
RNA	ribonucleic acid
RT	reverse transcription
S1P	site one protease
S2P	site two protease
SCAP	sterol-regulatory element-binding protein cleavage activator protein
SNP	single-nucleotide polymorphism
SRE	sterol regulatory element
SREBP	sterol-regulatory element-binding protein
TBE	tris-borate ethylenediaminetetraacetate
Tet	tetracycline
$TetO_2$	tetracycline operator 2
TNF-α	tumor necrosis factor-alpha
T-REx	tetracycline-regulated mammalian expression
Tris	tris(hydroxymethyl)aminomethane
UTR	untranslated region
wt	wild type

Summary

Human cells maintain lipid homeostasis by regulated cleavage of membrane-bound transcription factors, so-called sterol-regulatory element-binding proteins (SREBPs). The mature forms of SREBP-1 and -2 are transcriptional activators of lipogenic genes controlling cholesterol, fatty acids, and triglyceride biosynthesis and uptake. As the SREBPs play a central role in the regulation of the lipoprotein metabolism, we supposed that specific sequence variations, which correspond to single-nucleotide polymorphisms in these genes, and certain drugs, that influence the expression of SREBP, may result in alterations in plasma lipoprotein concentrations.

A syndrome characterized by hypertriglyceridemia, hypercholesterolemia, hyperinsulinemia and lipodystrophy has been found to be associated with antiretroviral treatment (ART) including protease inhibitors. A marker predicting this syndrome has been identified in the gene encoding the sterol-regulatory element-binding protein-1c (SREBP-1c), a regulator of triglycerides, cholesterol, insulin and adipocytes. A possible inhibition of SREBP-1c-dependent genes by the protease inhibitor indinavir and its possible reversal by the lipid-lowering drug simvastatin were studied in cell culture. The effects of indinavir and simvastatin on SREBP-1c-dependent genes were compared with the effects of indinavir and simvastatin on SREBP-1c-independent genes. In fact, indinavir inhibited the SREBP-1c-dependent genes encoding the lipoprotein lipase and the fatty acid synthase in a dose-dependent manner but not the SREBP-1c-independent gene encoding the low-density lipoprotein receptor. Furthermore, simvastatin antagonized the indinavir-induced SREBP-1c-inhibition. Thus, indinavir inhibits important effector genes of the SREBP-1c pathway, which may explain major antiretroviral treatment-related adverse effects.

A single-nucleotide polymorphism (3'322C/G SNP) identified in the sterol-regulatory element-binding protein-1c (SREBP-1c) gene was predictive of highly active antiretroviral therapy-related hyperlipoproteinemia. Increases in cholesterol, triglyceride and insulin were less frequently associated with homozygous SREBP-1c-3'322G (genotype 22) than with

heterozygous/homozygous SREBP-1c-3'322C (genotypes 11/12). The differences in messenger RNA conformation can explain the pharmacogenetic basis of these findings. The mRNA stability of both homozygous genotypes of SREBP-1c-3'322C/G was compared in the stably transfected T-REx cell lines using a real-time quantitative polymerase chain reaction method. The mRNA of the SREBP-1c-3'322C isoform (genotype 11) was shown to have a more abundance decay rate than 3'322G isoform (genotype 22). Thus, the sequence variation (3'322C/G SNP) in the coding 3' end of the gene affects the secondary structure of the SREBP-1c mRNA, influences its degradation rate and, therefore, causes differences in the regulation of SREBP-1c expression.

In the process of this thesis, three new splice variants of the human SREBP-1 gene that shared different combinations of the SREBP-1a and -1c exons at the 3' end of mRNA were identified. The splice variant containing exons 17, 18a and 18c was designated as SREBP-1d, the splice variant containing exons 17, 18a, 18c and 19c was termed as SREBP-1e, and the splice variant containing exons 17, 18c and 20f was named as SREBP-1f. Analysis of tissue distribution showed that the new splice variants SREBP-1e and -1d were ubiquitously found in various human tissues and tumor-derived cells, whereas wild-type SREBP-1c and SREBP-1f transcripts were relatively tissue-specific. This high abundance led us to the hypothesis that splice variants SREBP-1e and SREBP-1d play a more general role in regulating cellular lipid levels as compared to other isoforms.

This thesis concludes that the sterol-regulatory element-binding protein (SREBP)-1c is crucial in the metabolic side-effects associated with highly active antiretroviral therapy using protease inhibitors. Moreover, regulation mechanism mediated by the transcription factor SREBP-1 is a model of a complex gene regulation system composed of different related levels: promoter regulation of effector genes, differences in mRNA stability and tissue specific splice variants in different quantities.

I. Introduction

I.1. Molecular characterization of sterol-regulatory element-binding proteins (SREBPs)

Mammalian cells maintain their cholesterol content within a narrow range by controlling the rate of intracellular *de novo* cholesterol biosynthesis and the amount of cholesterol obtained from extracellular lipoproteins by receptor-mediated endocytosis (Brown and Goldstein, 1997; Wang *et al.*, 1994). Cholesterol can be taken up with the diet (exogenous pathway) or can be synthesized *de novo* (endogenous pathway). In mammals, cholesterol is mainly synthesized in the liver and in the intestinal mucosa. Apart from biosynthesis and receptor-mediated endocytosis, cholesterol homeostasis is also regulated by the rate of intestinal absorption and catabolic degradation in the liver (Chawla *et al.*, 2001). Further important physiological functions of cholesterol, especially for higher vertebrates, consist of its precursor role for the biosynthesis of, both, steroid hormones and bile acids. In eukaryotes, cholesterol is the main modulator of membrane fluidity and hence regulates phase transitions within the membrane (Bloch, 1983; Yeagle, 1985).

Animal cells regulate their biosynthetic pathways so as to produce the required amounts of end-products but, to prevent intracellular over-accumulation. An excess of intracellular cholesterol must be avoided as it forms toxic crystals whereas excess plasma cholesterol is deposited in arteries, initiating and accelerating atherosclerosis (Small and Shipley, 1974). End-product regulation of the cholesterol metabolism is achieved predominantly through transcriptional repression of genes that govern the synthesis of cholesterol and its receptor-mediated uptake from plasma lipoproteins (Goldstein and Brown, 1990). The synthesis and uptake of cholesterol in animal cells require membrane-bound transcription factors designated sterol-regulatory element-binding proteins (SREBPs) (Briggs *et al.*, 1993; Wang *et al.*, 1993).

SREBPs directly activate the expression of more than 30 genes dedicated not only to the synthesis and uptake of cholesterol, but also of fatty acids,

triglycerides, and phospholipids, as well as the NADPH cofactor required to synthesize these molecules (Brown and Goldstein, 1997; Goldstein *et al*., 2002; Sakakura *et al*., 2001). In the liver, SREBPs regulate the production of lipids for export into the plasma as lipoproteins and into the bile as micelles (Sheng *et al*., 1995).

I.1.1. Activation of SREBPs through proteolytic processing

SREBPs belong to the basic helix-loop-helix–leucine zipper (bHLH-Zip) family of transcription factors, but they differ from other bHLH-Zip proteins in that they are synthesized as inactive precursors bound to the endoplasmic reticulum (ER) (Brown and Goldstein, 1997; Goldstein *et al*., 2002). Each SREBP precursor of about 1150 amino acids is organized into three domains: (a) an NH_2-terminal domain of about 480 amino acids that contains the bHLH-Zip region for binding DNA; (b) two hydrophobic transmembrane–spanning segments interrupted by a short loop of about 30 amino acids that projects into the lumen of the ER; and (c) a COOH-terminal domain of about 590 amino acids that performs the essential regulatory function (Figure 1).

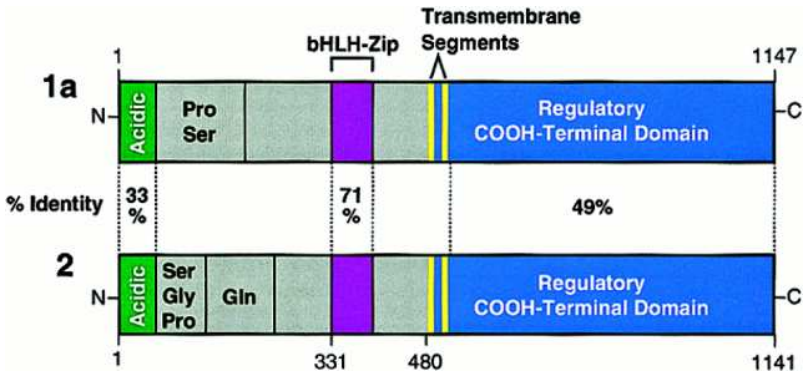


Figure 1. Domain structures of human SREBP-1a and SREBP-2. The sequence of SREBP-1c (not shown) is identical to that of SREBP-1a except for a shortened NH_2-terminal acidic domain (24 amino acids in SREBP-1c versus 42 amino acids in SREBP-1a) (Brown and Goldstein, 1997).

In order to enter the nucleus and act as a transcription factor, the NH_2-terminal domain of each SREBP must be released from the membrane proteolytically

(Figure 2). Three proteins required for SREBP processing have been delineated in cultured cells, using the tools of somatic cell genetics (Goldstein *et al*., 2002). One is an escort protein designated SREBP cleavage–activating protein (SCAP). The other two are proteases, designated Site-1 protease (S1P) and Site-2 protease (S2P). Newly synthesized SREBP is inserted into the membranes of the ER, where its COOH-terminal regulatory domain binds to the COOH-terminal domain of SCAP. SCAP is both an escort for SREBPs and a sensor of sterols. When cells become depleted in cholesterol, SCAP escorts the SREBP from the ER to the Golgi apparatus, where the two proteases reside. In the Golgi apparatus, S1P, a membrane-bound serine protease, cleaves the SREBP in the luminal loop between its two membrane-spanning segments, dividing the SREBP molecule (Figure 2). The NH_2-terminal bHLH-Zip domain is then released from the membrane via a second cleavage mediated by S2P, a membrane-bound zinc metalloproteinase. The NH_2-terminal domain, designated nuclear SREBP (nSREBP), enters the nucleus through nuclear lamin containing envelope (Caron *et al*., 2003) and activates transcription by binding to nonpalindromic sterol response elements (SREs) in the promoter/enhancer regions of multiple target genes. The binding of the NH_2-terminal domain of SREBP to specific promoter elements results in an increase in the LDL receptor-mediated uptake of LDL particles from the plasma and in an increase in the endogenous cholesterol biosynthesis.

When the cholesterol content of cells rises, SCAP senses the excess cholesterol through its membranous sterol-sensing domain, changing its conformation in such a way that the SCAP/SREBP complex is no longer incorporated into ER transport vesicles. The net result is that SREBPs lose their access to S1P and S2P in the Golgi apparatus, so their bHLH-Zip domains cannot be released from the ER membrane, and the transcription of target genes ceases (Horton *et al*., 2002). As a result, the synthesis of cholesterol and fatty acids declines.

Very recently it has been found the new component of SREBP pathway - Insig proteins. Under conditions of sterol excess, the SCAP/SREBP complex binds to Insig, an intrinsic membrane proteins of the ER (Yang *et al*., 2002; Yabe *et al*., 2002). This binding prevents the SCAP/SREBP complex from being incorporated into transport vesicles. As a result, SREBPs remain trapped in

the ER, and proteolytic processing cannot occur. The nuclear content of SREBPs declines rapidly as a result of proteasomal degradation. As a result, the synthesis of cholesterol and fatty acids declines. This finding confirms the crucial role of the SCAP sterol-sensing domain and its interaction with the Insig proteins for the maintenance of cellular cholesterol homeostasis.

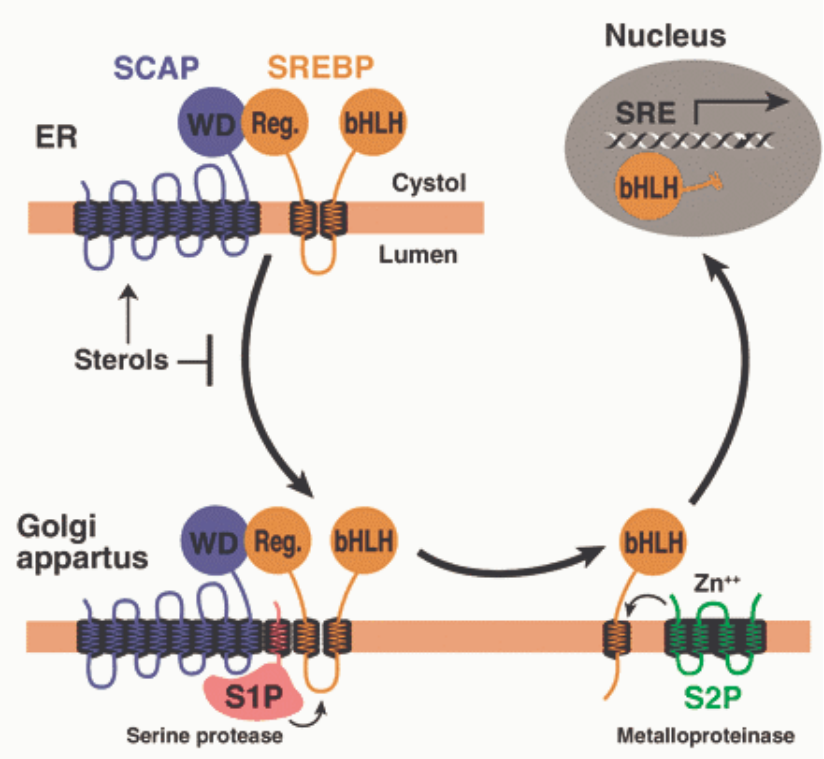


Figure 2. Model for the sterol-mediated proteolytic release of SREBPs from membranes. SCAP is a sensor of sterols and escorts the SREBPs. When cells are depleted of sterols, SCAP transports SREBPs from the ER to the Golgi apparatus, where two proteases, Site-1 protease (S1P) and Site-2 protease (S2P), act sequentially to release the NH_2-terminal bHLH-Zip domain from the membrane. The bHLH-Zip domain enters the nucleus and binds to a sterol response element (SRE) in the enhancer/promoter region of target genes, activating their transcription. When cellular cholesterol rises, the SCAP/SREBP complex is no longer incorporated into ER transport vesicles, SREBPs no longer reach the Golgi apparatus, and the bHLH-Zip domain cannot be released from the membrane. As a result, transcription of all target genes declines (Horton *et al.*, 2002).

When cholesterol is added to membrane vesicles *in vitro*, SCAP undergoes a conformational change as revealed by its differential sensitivity to proteolytic digestion (Brown *et al*., 2002). The correlation between this cholesterol-induced conformational change *in vitro*, and the binding of SCAP and the Insig proteins *in vivo*, indicates the possibility that the interaction between the two proteins requires SCAP to be in a conformation that depends on the presence or absence of specific lipids in the bilayer. The conformational change in SCAP could be secondary to the cholesterol-induced interaction between the Insig proteins and the SCAP sterol-sensing domain.
These results indicate a mechanism by which a conformational change in the sterol-sensing domain of SCAP converts information about the physical properties of membranes into an action that governs the transcription of genes with end products that ultimately alter the membrane composition.

I.1.2. The isoforms of SREBP

The mammalian genome encodes three SREBP isoforms, designated SREBP-1a, SREBP-1c, and SREBP-2. SREBP-2 is encoded by a gene on human chromosome 22q13. Both SREBP-1a and -1c are derived from a single gene on human chromosome 17p11.2 through the use of alternative transcription start sites that produce alternate forms of exon 1, designated 1a and 1c (Brown and Goldstein, 1997). SREBP-1a is a potent activator of all SREBP-responsive genes, including those that mediate the synthesis of cholesterol, fatty acids, and triglycerides. High-level transcriptional activation is dependent on exon 1a, which encodes a longer acidic transactivation segment than does the first exon of SREBP-1c. The roles of SREBP-1c and SREBP-2 are more restricted than that of SREBP-1a. SREBP-1c preferentially enhances transcription of genes required for fatty acid synthesis but not cholesterol synthesis. Like SREBP-1a, SREBP-2 has a long transcriptional activation domain, but it preferentially activates cholesterol synthesis (Brown and Goldstein, 1997). SREBP-1a and SREBP-2 are the predominant isoforms of SREBP in most cultured cell lines, whereas SREBP-1c and SREBP-2 predominate in the liver and most other intact tissues (Shimomura *et al*., 1997a).

When expressed at higher than physiologic levels, each of the three SREBP isoforms can activate all enzymes which used to generate cholesterol and fatty acids. However, at normal levels of expression, SREBP-1c favors the fatty acid biosynthetic pathway and SREBP-2 favors cholesterologenesis. SREBP-2–responsive genes in the cholesterol biosynthetic pathway include those for the enzymes HMG-CoA synthase (Smith *et al*., 1988), HMG-CoA reductase (Reynolds *et al*., 1984), farnesyl diphosphate synthase (Ericsson *et al*., 1996), and squalene synthase (Guan *et al*., 1995). SREBP-1c–responsive genes include those for ATP citrate lyase (which produces acetyl-CoA) (Shimano *et al*., 1999), acetyl-CoA carboxylase (Lopez *et al*., 1996), fatty acid synthase (Bennett *et al*., 1995) and lipoprotein lipase (Shimomura *et al*., 1998). Other SREBP-1c target genes encode a rate-limiting enzyme of the fatty acid elongase complex, which converts palmitate to stearate (Moon *et al*., 2001); stearoyl-CoA desaturase, which converts stearate to oleate; and glycerol-3-phosphate acyltransferase, the first committed enzyme in triglyceride and phospholipid synthesis (Edwards *et al*., 2000). Finally, SREBP-1c and SREBP-2 activate genes required to generate NADPH, which is consumed at multiple stages in these lipid biosynthetic pathways (Shimomura *et al*., 1998a).

I.1.3. Function of individual SREBP isoforms *in vivo*

To study the functions of individual SREBPs in the liver, transgenic mice overexpressing truncated versions of SREBPs (nSREBPs) that terminate prior to the membrane attachment domain have been produced. These nSREBPs enter the nucleus directly, bypassing the sterol-regulated cleavage step. By studying each nSREBP isoform separately, it was determined their distinct activating properties, albeit when overexpressed at nonphysiologic levels.
Overexpression of nSREBP-1c in the liver of transgenic mice produces a triglyceride-enriched fatty liver with no increase in cholesterol (Shimano *et al.*1997a). mRNAs for fatty acid synthetic enzymes and rates of fatty acid synthesis are elevated fourfold in this tissue, whereas the mRNAs for cholesterol synthetic enzymes and the rate of cholesterol synthesis are not increased (Shimomura *et al*., 1998a). Conversely, overexpression of

nSREBP-2 in the liver increases the mRNAs encoding all cholesterol biosynthetic enzymes; the most dramatic is a 75-fold increase in HMG-CoA reductase mRNA (Horton *et al.,* 1998). mRNAs for fatty acid synthesis enzymes are increased to a lesser extent, consistent with the *in vivo* observation that the rate of cholesterol synthesis increases 28-fold in these transgenic nSREBP-2 livers, while fatty acid synthesis increases only fourfold. This increase in cholesterol synthesis is even more remarkable when one considers the extent of cholesterol overload in this tissue, which would ordinarily reduce SREBP processing and essentially abolish cholesterol synthesis.

The consequences of overexpressing SREBP-1a, which is expressed only at low levels in the livers of adult mice, rats, hamsters, and humans have also been studied (Shimomura *et al*., 1997a). nSREBP-1a transgenic mice develop a massive fatty liver engorged with both cholesterol and triglycerides (Shimano *et al.,* 1996), with heightened expression of genes controlling cholesterol biosynthesis and, still more dramatically, fatty acid synthesis. The preferential activation of fatty acid synthesis (26-fold increase) relative to cholesterol synthesis (fivefold increase) explains the greater accumulation of triglycerides in their livers.

Considered together, the overexpression studies indicate that both SREBP-1 isoforms show a relative preference for activating fatty acid synthesis, whereas SREBP-2 favors cholesterol.

Knockout mice that lack all nSREBPs die early in embryonic development. For instance, a germline deletion of *S1P*, which prevents the processing of all SREBP isoforms, results in death before day 4 of development (Yang *et al.,* 2001; Mitchell *et al.,* 2001). Germline deletion of *SREBP-2* leads to 100% lethality at a later stage of embryonic development than does deletion of *S1P* (embryonic day 7–8). In contrast, germline deletion of *SREBP-1*, which eliminates both the 1a and the 1c transcripts, leads to partial lethality, in that about 15–45% of *SREBP-1*$^{-/-}$ mice survive (Shimano *et al.,* 1997a). The surviving homozygotes manifest elevated levels of SREBP-2 mRNA and protein, which presumably compensates for the loss of SREBP-1a and -1c. When the SREBP-1c transcript is selectively eliminated, no embryonic lethality is observed, suggesting that the partial embryonic lethality in the

SREBP-1$^{-/-}$ mice is due to the loss of the SREBP-1a transcript (Liang *et al.,* 2002).

The studies in genetically manipulated mice clearly show that, as in cultured cells, SCAP and S1P are required for normal SREBP processing in the liver. SCAP, acting through its sterol-sensing domain, mediates feedback regulation of cholesterol synthesis.

The SREBPs play related but distinct roles: SREBP-1c, the predominant SREBP-1 isoform in adult liver, preferentially activates genes required for fatty acid synthesis, while SREBP-2 preferentially activates the LDL receptor gene and various genes required for cholesterol synthesis. SREBP-1a and SREBP-2, but not SREBP-1c, are required for normal embryogenesis.

I.1.4. Transcriptional regulation of SREBP genes

Regulation of SREBPs occurs at two levels - transcriptional and posttranscriptional. The posttranscriptional regulation discussed above involves the sterol-mediated suppression of SREBP cleavage, which results from sterol-mediated suppression of the movement of the SCAP/SREBP complex from the ER to the Golgi apparatus (Figure 2). This form of regulation is manifest not only in cultured cells (Brown and Goldstein, 1997), but also in the livers of rodents fed cholesterol-enriched diets (Shimomura *et al.,* 1997).

The transcriptional regulation of the SREBPs is more complex. SREBP-1c and SREBP-2 are subject to distinct forms of transcriptional regulation, whereas SREBP-1a appears to be constitutively expressed at low levels in liver and most other tissues of adult animals (Shimomura *et al*., 1997a). One mechanism of regulation shared by SREBP-1c and SREBP-2 involves a feed-forward regulation mediated by SREs present in the enhancer/promoters of each gene (Sato *et al.,* 1996; Amemiya-Kudo *et al.,* 2000). Through this feed-forward loop, nSREBPs activate the transcription of their own genes. In contrast, when nSREBPs decline, as in *SCAP* or *S1P* knockout mice, there is a secondary decline in the mRNAs encoding SREBP-1c and SREBP-2 (Matsuda *et al.,* 2001; Yang *et al.,* 2001).

I.1.5. SREBPs in metabolic disorders and human disease

Many individuals with obesity and insulin resistance also have fatty livers, one of the most commonly encountered liver abnormalities (Marchesini *et al.,* 2001). A subset of individuals with fatty liver go on to develop fibrosis, cirrhosis, and liver failure. Evidence indicates that the fatty liver of insulin resistance is caused by SREBP-1c, which is elevated in response to the high insulin levels (Shimomura *et al*., 1999a).

The incidence of coronary artery disease increases with increasing plasma LDL-cholesterol levels, which in turn are inversely proportional to the levels of hepatic LDL receptors. SREBPs stimulate LDL receptor expression, but they also enhance lipid synthesis (Brown and Goldstein, 1997), so their net effect on plasma lipoprotein levels depends on a balance between opposing effects (Figure 3).

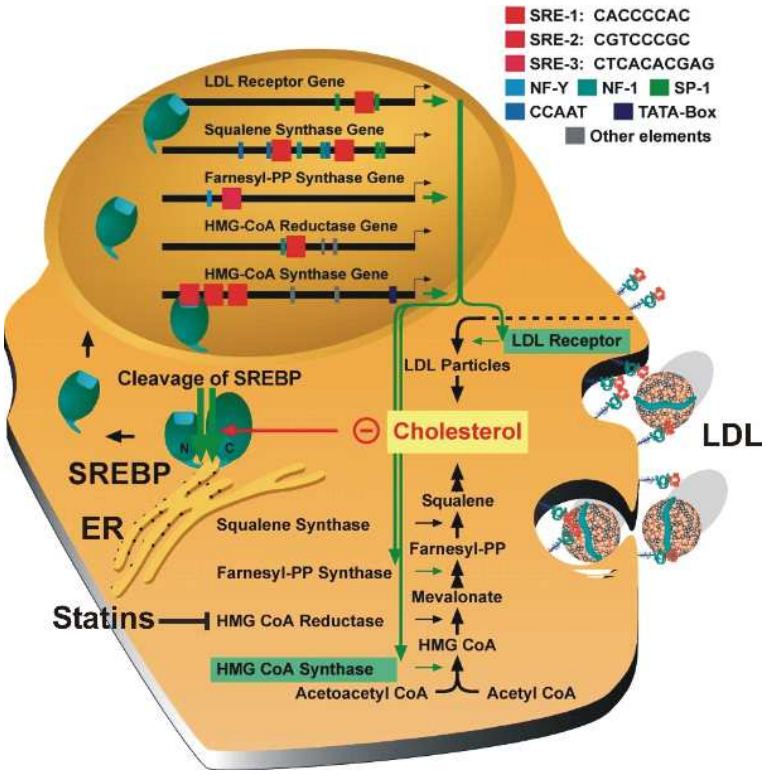


Figure 3. SREBP as a transcriptional enhancer of genes involved in the cholesterol uptake and biosynthesis. As the intracellular cholesterol concentration decreases, the active forms of the SREBPs bind to the sterol-regulatory elements (SREs) at the low-density lipoprotein (LDL) receptor promoter region and activate the LDL receptor gene (Südhof *et al*., 1987). Consequently, the number of LDL receptor molecules

available at the cell surface and thus, the receptor-mediated uptake of cholesterol-containing LDL particles increase. On the other hand, the SREBPs bind also to the promoters of genes involved in cholesterol synthesis and activate these genes. After administration of statins, the SREBP mediate the increase in the LDL receptor-mediated cholesterol uptake. The parallel SREBP-mediated increase in the cholesterol synthesis is inhibited by HMG-CoA reductase inhibitors, so the net effect of statins administration is a decrease in the plasma LDL cholesterol concentration.

The lowering of serum cholesterol is increasingly recognized as essential in the prevention of coronary heart disease and other atherosclerotic disease (Hebert *et al*., 1997). A class of widely used cholesterol-lowering drugs, the hydroxy-methyl-glutaryl-coenzyme A (HMG-CoA) reductase inhibitors, also called statins, inhibit the rate-limiting enzyme of the intracellular cholesterol synthesis, the HMG-CoA reductase (Garg and Grundy, 1988).

As has been outlined before, SREBPs play a central role in the regulation of the lipoprotein metabolism. Therefore specific sequence variations (e.g. corresponding to single-nucleotide polymorphisms) in the genes encoding the SREBPs and certain drugs, that influence the expression of SREBP, may result in alterations in plasma lipoprotein concentrations. Recently it has been reported that single-nucleotide polymorphisms in the SREBP-2 (A595G; R371K) and SREBP-1a (-36del/G) genes influence lipid parameters and the risk of coronary atherosclerosis (Muller and Miserez, 2002; Miserez *et al*., 2002; Vedie *et al*., 2001).

In this thesis we focussed on specific sequence variation of the human SREBP-1c gene and its influence on lipoprotein metabolism. We also analyzed the expression of major mediator genes of the lipid homeostasis upon administration of the HIV-protease inhibitor indinavir and explain the putative molecular mechanisms responsible for these adverse effects.

I.2. The role of SREBP-1c in drug induced hyperlipidaemia

I.2.1. The effects of HIV-protease inhibitor indinavir on SREBP-1c

In the recent years antiretroviral treatment (ART) including protease inhibitors (PIs) has drastically lowered morbidity and immediate mortality in HIV-1-infected patients (Egger *et al*., 1997; Palella *et al*., 1998). Currently, the recommended therapy for such patients includes the use of one or two HIV protease inhibitors (PIs) combined with two nucleoside reverse transcriptase inhibitors (NRTIs) or two nucleoside RTIs combined with one non-nucleoside reverse transcriptase inhibitor (NNRTI) (Carpenter *et al*., 2000). Inhibition of the HIV protease prevents cleavage and maturation of the viral polyprotein precursor leading to production of non-infectious viral particles (Flexner, 1998). The HIV reverse transcriptase is required to copy the viral RNA genome and inhibitors used to target this enzyme consist of non-nucleoside, non-competitive inhibitors or chain-terminating nucleoside analogues (Beach, 1998).

The problem of this highly active antiretroviral therapy is the development of hypertriglyceridemia, hypercholesterolemia, hyperinsulinemia and lipodystrophy (Carr *et al*., 1998; Carr *et al*., 1999). Thus, PIs, although drastically lowering immediate morbidity and mortality, may increase the risk of cardiovascular complications (Henry *et al*., 1998; Périard *et al*., 1999). Recent studies revealed that ART-related adverse effects are common and persist in patients remaining on treatment (Carr *et al*., 1998, Périard *et al*., 1999).

The recent experiments show that sterol-regulatory element-binding protein -1 (SREBP-1), a transcription factor known to promote lipogenic gene expression, could be involved in this process. Dowell *et al.* observed that several HIV protease inhibitors inhibited preadipocyte differentiation and promote adipocyte cell death. The level of the proteolytically processed, active form of sterol-regulatory element-binding protein-1 was also reduced markedly in nelfinavir-treated cells, whereas the level of the precursor form of this protein was unaffected (Dowell *et al*., 2000). The results of Caron *et al.* show

that indinavir inhibit the adipocyte differentiation program of preadipocytes at an early step and impairs SREBP-1 intranuclear localization in cultured cells. The molecular mechanism of these clinical and biological alterations is currently unknown but it is probably multifactorial (Caron *et al.*, 2001).
To investigate the molecular basis responsible for these adverse effects, we firstly determined whether the key regulators of lipid homeostasis, SREBP-1c or SREBP-2, may be involved. Reporter gene assays with major effector genes of lipid metabolism were performed. To show a potential association the effect of the PI indinavir on SREBP-1c and the consequences of a possible inhibition was determined. Effector genes specifically activated by SREBP-1c in cell culture in the presence of indinavir were studied. Additionally the possible reversal by the lipid-lowering drug simvastatin was investigated. Finally, the effects of indinavir and simvastatin on the inhibition/activation of SREBP-1c-dependent genes were compared with the effects of indinavir and simvastatin on the SREBP-1c-independent genes.

I.2.2. The role of single-nucleotide polymorphism in SREBP-1c gene in protease inhibitor-induced hyperlipoproteinemia

Recent studies revealed that adverse effects (hyperlipoproteinemia, insulin resistance and lipodystrophy) of antiretroviral treatment (ART) affect only a part of the subjects treated with PIs (Carr *et al.*, 1999; Segerer *et al.*, 1999; Carr, 2000). Therefore, the presence of subjects without any increase in plasma lipoprotein concentrations or insulin resistance and diabetes mellitus, and of others with striking increases in lipoproteins and insulin suggests an underlying genetic predisposition to this syndrome.
Miserez *et al.* recently demonstrated that genetic variants of the sterol-regulatory element-binding protein-1c, also called adipocyte determination and differentiation factor 1 (SREBP-1c/ADD-1), can modify plasma cholesterol and triglyceride levels. After identification of a frequent single-nucleotide polymorphism (SNP), 3'322C/G, in the SREBP-1c gene, it was demonstrated that this inherited SNP predicts the development of PI-associated hyperlipoproteinemia. This single-nucleotide polymorphism does not alter the amino acid sequence. The frequency of SREBP-1c-3'322C homozygosity

(genotype 11) was 40.3%, SREBP-1c-3'322C/SREBP-1c-3'322G heterozygosity (genotype 12) 45.6%, and SREBP-1c-3'322G homozygosity (genotype 22) 14.1%, resulting in a high polymorphism information content value of 0.37. Increases in cholesterol were less frequently associated with homozygous SREBP-1c-3'322G (genotype 22) than with heterozygous/homozygous SREBP-1c-3'322C (genotypes 11/12) and correlated with leptin and insulin increases, particularly in genotype 11/12 carriers (Miserez *et al*., 2001; Dorenbaum and Miserez, 2001).

To further investigate the molecular basis of these SNP-associated differences, we first excluded the possibility that SREBP-1c-3'322G causes alternative splicing and used computer-based analyses to predict differences in the putative mRNA secondary structure (SREBP-1c-3'322C versus SREBP-1c-3'322G). Furthermore, synonymous mutations, such as SREBP-1c-3'322C/G, cannot be *a priori* considered as neutral or non-pathogenic, because they may directly affect mRNA structure and/or stability (Richard and Beckmann, 1995). This finding is particularly important because it has been demonstrated that the SREBP-1c pathway is regulated not only by cleavage activation but also by rate of mRNA degradation (Xu *et al*., 1999; Xu *et al*., 2001).

As the expression of the SREBP-1c gene can be regulated at the mRNA level, other specific sequence variations (3'322C/G SNP) could affect the secondary structure of the SREBP-1c mRNA and influence its degradation rate additionally. A possible association between the mRNA stability of the SREBP-1c gene in the presence of a single-nucleotide polymorphism 3'322C/G was determined *in vitro*. For that, the mRNA stability of both homozygous genotypes of SREBP-1c-3'322C/G was compared in the stably transfected T-REx cell lines using a real time quantitative polymerase chain reaction method.

I.3. Alternative splicing of human SREBP-1

The protein coding sequences of most eukaryotic messenger RNA precursors (pre-mRNAs) are interrupted by non-coding sequences called introns. Pre-

mRNA splicing is the process by which introns are removed and the protein coding elements assembled into mature mRNAs. Alternative pre-mRNA splicing selectively joins different protein coding elements to form mRNAs that encode proteins with distinct functions, and is therefore an important source of protein diversity (Maniatis and Tasic, 2002). The proteome of a cell can rapidly change in response to extracellular stimuli through complex signal-transduction pathways. Changes in protein composition can be regulated at many different levels: transcription, post-translational protein modification and inducible alternative pre-mRNA splicing. A recent study estimated that greater than 55% of human genes are alternatively spliced (Kan *et al.*, 2001). Although a number of different factors that affect splicing have been identified, the molecular mechanisms of alternative pre-mRNA splicing remain poorly defined (Maniatis and Tasic, 2002).

Sterol-regulatory element-binding protein-1 (SREBP-1) belongs to the family of the basic/helix-loop-helix/leucine zipper transcription factors. They have emerged as a major factor involved in the fatty acid, phospholipids, triglycerides and insulin regulation (Horton *et al.*, 2002). Both SREBP-1a and -1c isoforms are derived from a single gene on human chromosome 17p11.2 through the use of alternative splicing at both the 5' and the 3' ends of the mRNA. The gene spans 26 kb and encodes 22 exons and 20 introns. Exons 1a and 1c are transcribed from different promoters using two alternative transcription start sites and spliced to a common exon 2. Exons 2-17 have been found in all SREBP-1 cDNAs (Hua *et al.*, 1995). SREBP-1a and SREBP-1c differ in sequence at both of their 5' and 3' ends. Splice variants SREBP-1a and -1c are different in exons 1, 18 and 19 (Figure 4). One full-length human cDNA contained the c sequences on both ends and was designated SREBP-1c. The rat homologue of SREBP-1c, named ADD1 (adipocyte differentiation and determination factor one), was cloned independently as a protein which binds to E-boxes, and presumably promotes adipocyte differentiation (Tontonoz *et al.*, 1993). A full-length cDNA isolated from Chinese hamster ovary (CHO) cells contained the a sequences on both ends and was named SREBP-1a (Sato *et al.*, 1994). A cDNA containing the a sequence on the 5' end and the c sequence on 3' end was created artificially and was designated SREBP-1b (Yokoyama *et al.*, 1993).

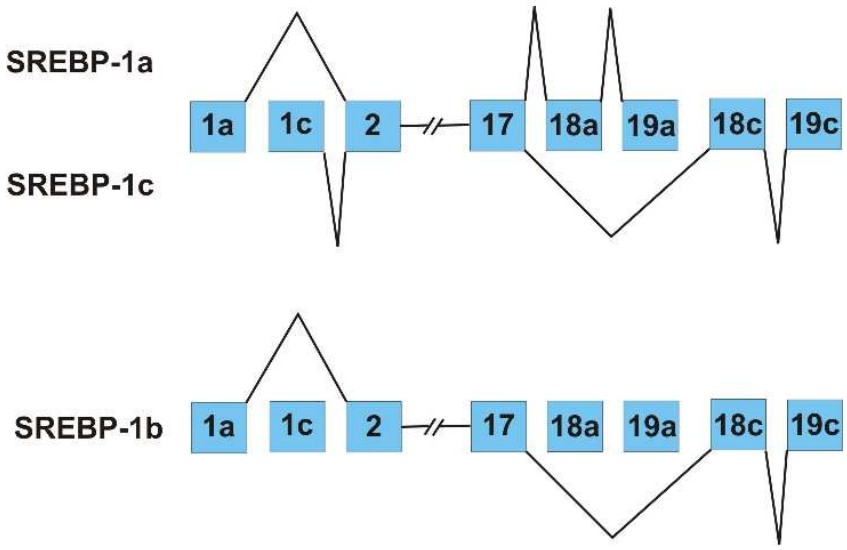


Figure 4. Alternative splicing of the SREBP-1 gene. The splicing patterns that produce three alternatively spliced transcripts, SREBP-1a, SREBP-1b and SREBP-1c, are indicated.

SREBP-1a is a potent activator of all SREBP-responsive genes, including those that mediate the synthesis of cholesterol, fatty acids, and triglycerides. High-level transcriptional activation is dependent on exon 1a, which encodes a longer acidic transactivation segment than does the first exon of SREBP-1c. SREBP-1c preferentially enhances transcription of genes required for fatty acid synthesis but not cholesterol synthesis. SREBP-1a is the predominant isoform of SREBP in most cultured cell lines, whereas SREBP-1c predominates in the liver and most other tissues (Shimomura *et al.*, 1997a).
In the process of cloning the SREBP-1c gene we observed a number of different mRNA transcripts indicating possible alternative splicing of this gene in humans at the 3' end of pre-mRNA. We analyzed these transcripts using reverse transcriptase (RT) experiments in human normal and tumor tissues, in lymphocytes from several patients, and, finally, identified them by sequencing.

III. Materials and Methods

III.1. Materials

III.1.1. Cell culture reagents and chemicals

Cholesterol, 25-hydroxycholesterol, poly-D-lysine hydrobromide, lipoprotein-deficient serum (LPDS), penicillin, streptomycin, actinomycin D and ethidium bromide were purchased from Sigma, St. Louis, MO, USA. Cell culture media supplements were obtained from Invitrogen (Invitrogen, Carlsbad, CA, USA). Agarose was obtained from Applichem (Applichem, Darmstadt, Germany). Indinavir sulfate and simvastatin were provided by Merck & Co. Inc., Rahway, New Jersey, USA. The inactive lactone prodrug simvastatin was converted into its active dihydroxy-open form (L-644128) by acidic hydrolysis according to the manufacturer's protocol. Aqueous solutions of indinavir sulfate and the active dihydroxy-open form of simvastatin were prepared. All other chemicals used were of best quality available and were purchased from Merck (Merck, Darmstadt, Germany), Sigma or Fluka (Fluka, Buchs, Switzerland).

III.1.2. Buffers

Buffers were purchased from Invitrogen.

Name	Composition
TAE buffer 50x	2 M Tris/acetate, pH 8.5, 50 mM EDTA
TBE buffer 10x	1 M Tris, 0.9 M Boric acid, 10 mM EDTA, pH 8.4
TE buffer 1x	10 mM Tris/HCl, pH 8.0, 1 mM EDTA

III.1.3. PCR primers and oligonucleotides

Primers were obtained from Microsynth (Microsynth, Balgach, Switzerland).

Name	Sequence
GAPDH F primer	5'-ACATCATCCCTGCCTCTACTGG-3'
GAPDH R primer	5'-AGTGGGTGTCGCTGTTGAACTC-3'
FAS F primer	5'-GGAGGTACCGCGTTCCTTGTGCTCCAGCGCGC-3'
FAS R primer	5'-CCCAAGCTTCGCGTCCCTCTGGAGGAGCTG CAA G-3'

LDLR F primer	5'-AGCTGGTACCCGGAGACCCAAATACAACA-3'
LDLR R primer	5'-TGTCCAAGCTTGAAACCCTGGCTTCCCGCGA-3'
LPL F primer	5'-GGGGTACCTGCAGGAGTATTCTATATAAGATAG-3'
LPL R primer	5'-CCCAAGCTTCGCGTCCCTCTGGAGGAGCTGCAAG-3'
SREBP-1c primer F	5'-CCCAAGCTTGGAGGGGTAGGGCCAACGGCCTGGAC-3'
SREBP-1c primer R	5'-CCGCTCGAGGTTTCTAAAAGATGTTTATTTTCCTTAAG-3'
SREBP-1c-3322C/G F	5'-CCCTCCCCCAGCACCTAGGGAAAGGCTTCCCCTCCCCC-3'
SREBP-1c-3322C/G R	5'-GGGGGAGGGGAAGCCTTTCCCTAGGTGCTGGGGGAGGG-3'
SREBP-1a primer F	5'-GATCTGGACGCGGCGCTGCTGACCGACATCG-3'
F ex 17 primer	5'-AGGCCCCAGGCTTCCGCCCTTGAGC-3'
R 3'UTR primer	5'-GGTGAGACGTGCCAGACTTCTTGCAG-3'
R ex 2 SREBP-1	5'-CATGGACGGGTACATCTTCAATGGAGTGG-3'
Random hexamer	$d(N)_6$

III.2. Methods

III.2.1. Cell culture

III.2.1.1. Growth and maintenance of cell lines

Human embryonic kidney (HEK) 293 and human hepatocellular carcinoma (HepG2) cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS) (Invitrogen), 100 units/ml penicillin, and 100 µg/ml streptomycin (Sigma). The confluent culture was splited 1:5 every 3 days using tripsin/EDTA solution, seeded into sterile culture flasks and cultured in an incubator at 37°C under 5% CO_2 atmosphere and saturated humidity. The cell lines were used at passage number 3-15 in all studies.

III.2.1.2. Freezing and thawing of cultured cells

For freezing only the early-passage cells were used. The freezing of multiple vials of the particular cell line ensure an adequate supply of early-passage cells. The cells were frozen in freezing medium (70% Dulbecco's modified Eagle's medium, 20% FBS, 10% DMSO) at a density of 5 x 10^6 cells/cryovial. Cryotubes were transferred into the freezing container (Nalgene, San Diego, CA, USA) and hold overnight at -70°C. For long storage, cryovials were transferred into liquid nitrogen.

For thawing the cryotube containing 5 x 10^6 cells/ml was removed from liquid nitrogen and thawed quickly at 37°C. Subsequently, the cells suspension was transferred to a sterile 50 ml Falcon tube (BD Falcon) containing 20 ml of complete medium and centrifuged for 10 minutes at 750 x g at room temperature to wash the DMSO away. The cells were resuspended in 25 ml fresh, complete medium, seeded on culture flasks and cultured in an incubator at 37°C under 5% CO_2 atmosphere and saturated humidity until the cells were 80-90% confluent.

III.2.2. Construction of plasmids

III.2.2.1. Lipoprotein lipase reporter gene construct

The pGL2-lipoprotein lipase (LPL) reporter gene construct contained nucleotides -1910 to -9 (A of the ATG translation start site was assigned +1 in all the constructs) of the LPL gene promoter (GenBank X68111; nucleotides 1 to 1902 according to GenBank numbering), comprising three putative sterol-regulatory elements. The promoter was amplified by polymerase chain reaction (PCR) using the oligonucleotide sequences 5'-GG**G GTA CC**T GCA GGA GTA TTC TAT ATA AGA TAG-3' and 5'-CCC **AAG CTT** CGC GTC CCT CTG GAG GAG CTG CAA G-3' (restriction sites are shown in bold), digested with *Kpn* I and *Xho* I (New England Biolabs, Beverly, MA, USA), purified with QIAquick purification kit (Qiagen, Valencia, CA, USA), and ligated using Takara ligation kit (Takara, Shiga, Japan) into the pGL2–Basic vector (Promega, Madison, WI, USA).

III.2.2.2. Fatty acid synthase reporter gene construct

The pGL2-fatty acid synthase (FAS) reporter gene construct contained nucleotides -1485 to -1246 of the FAS gene promoter (GenBank X54671; nucleotides 1381 to 1620 according to GenBank numbering), comprising the key regulatory elements such as the sterol-regulatory element. The promoter was cloned into pGL2 following PCR (5'-GGA **GGT ACC** GCG TTC CTT GTG

CTC CAG CGC GC-3'; 5'-CAG **AAG CTT** CTG GAC GGG ACG CTG CTG CCG TCT CTC-3') using *Kpn* I and *Xho* I restriction sites.

III.2.2.3. Low-density lipoprotein receptor (LDLR) reporter gene construct

The pGL2-LDLR-construct contained nucleotides -328 to -61 of the LDLR gene promoter (GenBank L29401; nucleotides 380 to 627 according to GenBank numbering), comprising one SRE. The promoter was cloned into pGL2 following PCR (5'-AGC T**GG TAC C**CG GAG ACC CAA ATA CAA CA-3'; 5'-TGT CC**A AGC TT**G AAA CCC TGG CTT CCC GCG A-3') using *Kpn* I and *Xho* I restriction sites.

III. 2.2.4. Cloning of human wild-type SREBP-1c

Expression plasmid for human SREBP-1c was constructed by cloning full-length coding sequences and the entire 3' untranslated region (3' UTR) of SREBP-1c using forward primer: 5'-CCC **AAG CTT** GGA GGG GTA GGG CCA ACG GCC TGG AC-3' and reverse primer 5'-CCG **CTC GAG** GTT TCT AAA AGA TGT TTA TTT TCC TTA AG-3' (restriction sites for *Xho* I and *Hind* III are shown in bold) from HeLa cDNA library. The amplicon was digested, purified, and ligated into the expression vector pcDNA3 (Invitrogen). Sequencing of SREBP-1c clones was done by Microsynth, single strand sequencing using vectors and internal sequencing primers.

III. 2.2.5. Cloning of human SREBP-1c containing single-nucleotide polymorphism 3'322C/G; site-directed mutagenesis

A plasmid pcDNA3 containing the wild-type SREBP-1c cDNA (pcDNA3-SREBP-1c) was used as a template for site-directed mutagenesis. The sequence variant discovered (bold) was introduced by using the oligonucleotides SREBP-1c-3'322C/G - F primer: 5'-CCC TCC CCC AGC ACC TAG G**G**A AAG GCT TCC CCT CCC CC-3', SREBP-1c-3'322C/G - R primer (its inverse complementary sequence) and the QuikChange$^{(TM)}$ Site-

Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA). *Epicurian Coli* XL1-Blue supercompetent cells were transformed according to the instructions of the manufacturer. Fifty clones were PCR-amplified using primers homologous to exon 17 and 3'-untranslated region of SREBP-1c (F ex17 primer: 5'-AGG CCC CAG GCT TCC GCC CTT GAG C-3' and R 3'UTR primer: 5'-GGT GAG ACG TGC CAG ACT TCT TGC AG-3', respectively). The amplicons were digested with *Xmn* I (New England Biolabs) to confirm the presence of the sequence encoding 3'322G. Positive clones were sequenced. The modified region containing the sequence encoding 3'322G was cut out using *Xho* I and *Bsu36* I (New England Biolabs) and ligated into the corresponding sites of the parental plasmid pcDNA3-SREBP-1c. The presence of the nucleotide substitution encoding the 3'322G isoform was again confirmed by sequencing (Microsynth).

III. 2.2.6. Cloning of human alternative splice variant SREBP-1d

Expression plasmid for human SREBP-1d was constructed by cloning the 3'-region of coding sequences of SREBP-1 from a liver cDNA library. For PCR amplification the forward (F ex17) primer 5'-AGG CCC CAG GCT TCC GCC CTT GAG C-3' and reverse (SREBP-1c R) primer 5'-CCG **CTC GAG** GTT TCT AAA AGA TGT TTA TTT TCC TTA AG-3' (restriction site for *Xho* I is shown in bold) were used. The amplicon was digested using *Xho* I and *Bsu36* I restriction endonucleases (New England Biolabs), purified with QIAquick purification kit (Qiagen) and ligated into the corresponding sites of the expression vector pcDNA3-SREBP-1c.

The 5'-end of the splice variant SREBP-1d was determined by polymerase chain reaction using two forward primers homologous to exon 1a (SREBP-1a F primer: 5'-GAT CTG GAC GCG GCG CTG CTG ACC GAC ATC G-3') and exon 1c (SREBP-1c F primer: 5'-CCC AAG CTT GGA GGG GTA GGG CCA ACG GCC TGG AC-3'), and reverse primer homologous to exon 2 (R ex 2 SREBP-1 primer: 5'-CAT GGA CGG GTA CAT CTT CAA TGG AGT GG-3') which is specific for SREBP-1a and SREBP-1c. Liver cDNA library expressing only the SREBP-1d splice variant was used as a template. The polymerase chain reaction analysis revealed that the amplification occurs only when the

forward primer, homologous to exon 1c of the SREBP-1c isoform, was used. Therefore, the splice variant SREBP-1d contains at the 5'-end the exon 1c and the N-terminus of this splice variant is similar to the SREBP-1c isoform.

III.2.2.7. Confirmation of constructs

All constructs were confirmed by sequencing and contained sequences identical to those previously published and demonstrated to be functional (Schoonjans *et al*., 2000; Bennett *et al*., 1995; Shimano *et al*., 1997; Hua *et al*., 1993). In the reporter gene experiments, the regulatory sequences of the inserts have been demonstrated to be inhibited by sterols as a negative and activated by simvastatin as a positive control.

III.2.3. Dual-Luciferase Reporter assay

III.2.3.1. Principle of the Dual-Luciferase Reporter assay system

The Dual-Luciferase Reporter (DLR) assay (Promega) is an advanced co-reporter technology integrating firefly (*Photinus pyralis*) and sea pansy (*Renilla reniformis*) luciferase assays. Although both the firefly and *Renilla* luciferases provide the superior assays characteristics of bioluminescent reporters, they possess distinct evolutionary origins and, therefore, have dissimilar enzyme structures and substrate requirements.

Firefly luciferase is a monomeric 61 kDa protein that does not require post-translational processing for enzymatic activity. Thus, it functions as a genetic reporter immediately upon translation. Photon emission occurs via oxidation of beetle luciferin in a reaction that requires ATP, Mg^{2+} and O_2.

Renilla luciferase, a monomeric 36 KDa protein, is composed of 3% carbohydrate when purified from its natural source, *Renilla reniformis*. However, like firefly luciferase, post-translational modification is not required for activity and enzyme may function as a genetic reporter immediately following translation. The luminescent reaction catalyzed by *Renilla* luciferase utilizes O_2 and coelenterate luciferin (coelenterazine).

In the DLR Assay System, the activities of the firefly and *Renilla* luciferases are measured sequentially from a single lysate. Upon completing the measurement of firefly luciferase activity (the 'experimental' reporter), the firefly luminescence is rapidly quenched, with simultaneous activation of the *Renilla* luciferase luminescent reaction (the 'control' reporter activity). Thus, the DLR Assay System integrates the two assay chemistries to provide rapid quantitation of both reporters co-expressed in the lysates of transfected cells.

The promoter region of a gene regulated by transcription factor of interest (SREBP) is cloned into pGL-vector polylinker (Promega) in front of the firefly luciferase gene (Figure 5). The enzymatic activity of the luciferase as reporter gene in transfected HEK-293 cells is directly proportional to its transcriptional activation which in turn is proportional to the transcriptional activity of the transcription factor (SREBP) and is normalized with the *Renilla* luciferase internal control constitutively expressed from pRL-CMV (Promega).

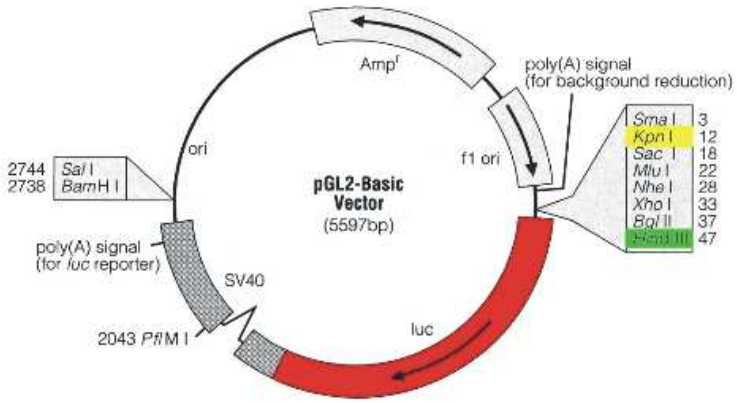


Figure 5. pGL2-Basic Vector (Promega).

Measurement of firefly and *Renilla* luciferases was performed on a Microlumat Plus according to the manuals of the luminometer (LB 96V EG & G Berthold, Bad Wildbad, Germany) and of the kit. The firefly luciferase reporter assay was initiated by injecting 50 µl of the Luciferase Assay Reagent II (LAR II) to the 20 µl lysate into 96-well plate. After a delay time 1 s RLU-measurement was integrated during 5 s (with subtraction of background). Upon completion of the firefly luciferase assay, the firefly luminescence is quenched and *Renilla* luminescence is simultaneously activated by injecting 50 µl of the Stop & Glo

Reagent to the sample well. After delay time of 1 s RLU-measurement was integrated during 5 s (without subtraction of back ground). The Stop & Glo Reagent quenches the luminescence signal from the firefly reaction by >105-fold within 1 s. The simultaneous activation of *Renilla* luciferase is also achieved within this period.

III.2.3.2. Setup for the Dual-Luciferase Reporter assay

Monolayers of human embryonic kidney (HEK)293 and hepatoma (Hep)G2 cells were set up (day 0, 5 × 10^6 cells/100 mm poly-D-lysine-coated dish) and cultured (37°C, 5% CO_2) in Dulbecco's Modified Eagle Medium (DMEM) (Invitrogen) supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin (Sigma) and 5% (v/v) fetal bovine serum (FBS) (Invitrogen) for 15 h. Transfection was carried out using GenePorter2 Reagent kit (Gene Therapy Systems, San Diego, CA, USA) according to the manufacturer's directions. Luciferase reporter assays were performed by co-transfection with 8.4 µg luciferase reporter gene constructs and 0.2 µg pRL-CMV (Promega), a plasmid encoding the *Renilla* luciferase, as an internal control for transfection efficiency into HEK293 cells seeded in 100 mm dishes. The cells were incubated for 7 h, trypsin-treated, and transferred to medium A (DMEM supplemented with 5% (v/v) FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin) or medium B (DMEM supplemented with 5% (v/v) calf lipoprotein-deficient serum (LPDS) (Sigma), 100 U/ml penicillin, and 100 µg/ml streptomycin). Than the cells were distributed over 96-well plates and incubated for 17 h. Each well contained 2x10^4 viable cells.

Twenty-four hours after transfection, certain drugs or active compounds were added to indicated final concentrations. As a control of the inhibition of the SREBP-regulated reporter genes, the cells were incubated with 1 µg/ml 25-hydroxycholesterol and 10 µg/ml cholesterol (Sigma); as a control of the activation of the SREBP-regulated reporter genes, the cells were incubated with the dihydroxy-open form of simvastatin at final concentrations of 3x10^4 or 4x10^4 nmol/l.

Following incubation for 24 h, the media were discarded, the cells were washed with 1x PBS, and passive lysis buffer (25 µl/well; Promega) was

added. The 96-well plates were shaken for 20 min. The luciferase and Renilla activities were determined by the Dual-Luciferase™ Reporter Assay System (Promega). Luciferase activities were normalized according to the Renilla activities. Luciferase activity was measured with a Microlumat Plus according to the manuals of the luminometer (LB 96V EG & G Berthold).

III. 2.4. Isolation of lymphocytes

To 7 ml Lymphodex (Gull Laboratories, Salt Lake City, UT, USA) 7 ml heparinized blood was added without mixing the phases. The tubs were centrifuged at 3500 x g for 10 min. The upper yellow phase (plasma) was discarded and the medium colorless phase (lymphocytes) was transferred to a tube containing 30 ml PBS. The tubs were centrifuged at 1000 x g for 10 min. The supernatant was discarded; the pellet was resuspended in 1 ml Trizol reagent (Invitrogen) and used for total RNA extraction.

III.2.5. Extraction of total cellular RNA from human lymphocytes and cultured cells

Total cellular RNA was isolated using Trizol reagent (Invitrogen) followed by the RNeasy total RNA isolation mini kit (Qiagen) according to the manufacturer's instructions. Cells were washed once rapidly with phosphate-buffered saline, lysed by adding 1.5 ml Trizol reagent to the plates, removed from the plates and transferred to Eppendorf tubes. Chloroform (300µl; Sigma) was added, the phases were mixed and centrifuged for 10 min at 13 000 x g. The superior layers were carefully removed and applied onto the RNeasy spin column. RNA was recovered in RNase-free water and quantified by spectrophotometry at 260 nm. The quality of the RNA was checked by examining ribosomal RNA bands after agarose gel electrophoresis and by amplifying GAPDH as a control. No RNA degradation due to storage was found.

III.2.6. Reverse transcription (RT)

cDNA was made by reverse transcribing 5 µg total RNA using the enzyme SuperScript II (Invitrogen) and random hexamers primers (Promega) according to the manufacturer's directions. DNase treatment was deemed unnecessary for quantitative PCR as the amplicons for GAPDH and SREBP-1 span intron/exon boundaries and genomic DNA contaminants would not amplify under the limiting thermocycling conditions used. The total RNA was first incubated with 100 ng of random hexamer primers (Promega) and 200 µM of dNTP (PeqLab, Erlangen, Germany) at 65°C for 7 minutes and then reverse-transcribed at 42°C for 1 hour in a reaction mix containing final concentration of 1x first strand buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$), 100 mM dithiothreitol, 200 units of SuperScript II Reverse Transcriptase, and 40 units of ribonuclease inhibitor RNAsin (Promega).

III.2.7. Polymerase chain reaction (PCR)

PCR was performed on a Perkin Elmer 9700 thermocycler with 100-200 ng of genomic DNA or 100 ng of cDNA obtained from the reverse transcription of total RNA. To the DNA template the following compounds were added: a set of primers (0.5 µM), 1.25 units of *Taq* DNA polymerase (Qiagen), 2.5 µl 10x PCR buffer (Qiagen), $MgCl_2$ at a final concentration of 1.5 µM, dNTP (PeqLab) at a final concentration of 200 µM and a water to a final volume of 25 µl. The reaction was incubated for 3 min at 95°C to activate *Taq* DNA polymerase in order to get a hot start PCR. Subsequently 35-39 cycles of 30 s denaturation at 94°C, 1 min annealing at a primer specific temperature (55 to 60°C) and 40 s to 5 min extension at 72°C were performed. After the last cycle, the reaction was maintained for 10 min at 72°C for terminal elongation. Semi-quantitative multiplex PCR for SREBP-1 and GAPDH cDNAs was done in 22-25 cycles in such a way that PCR amplification is still in log-linear phase.

The PCR and RT-PCR products were analyzed by 1-2% agarose gel electrophoresis, and band intensities were compared by imaging of ethidium bromide (0.5 µg/ml) staining.

III.2.8. Generating of stable tetracycline-inducible cell lines

III.2.8.1. Principle of the T-REx System

The T-Rex System is a tetracycline - regulated mammalian expression system that uses regulatory elements from the *E. coli* Tn*10*-encoded tetracycline (Tet) resistance operon (Hillen and Berens, 1994; Hillen *et al*., 1983). Tetracycline regulation in the T-REx System is based on the binding of tetracycline to the Tet repressor and derepression of the promoter controlling expression of the gene of interest (Yao *et al*., 1998). The major components of the T-REx System include: an inducible expression plasmid, pcDNA4/TO, for expression of a gene of interest under the control of the strong human cytomegalovirus immediate-early (CMV) promoter with two tetracycline operator 2 ($TetO_2$) sites and a regulatory plasmid, pcDNA6/TR, which encodes the Tet repressor (TetR) under the control of the human CMV promoter.

In the T-REx System, expression of our gene of interest is repressed in the absence of tetracycline and induced in the presence of tetracycline (Yao *et al*., 1998). Tetracycline-regulated gene expression in the T-REx System more closely resembles the regulation of the native bacterial tet operon (Hillen and Berens, 1994) and avoids the potentially toxic effects of viral transactivation domains observed in some mammalian cell lines.

The major component of the T-REx System is the inducible expression plasmid pcDNA4/TO. Expression of gene of interest from the inducible expression vector is controlled by the strong CMV promoter into which 2 copies of the tet operator 2 ($TetO_2$) sequence have been inserted in tandem. The $TetO_2$ sequences consist of 2 copies of the 19 nucleotide sequence, 5´-TCCCTATCAGTGATAGAGA-3´ separated by a 2 base pair spacer (Hillen and Berens, 1994). Each 19 nucleotide $TetO_2$ sequence serves as the binding site for 2 molecules of the Tet repressor. The second major component of the T-REx System is the pcDNA6/TR regulatory vector which expresses high levels of the TetR gene (Postle *et al*., 1984) under the control of the human CMV promoter. Both T-REx vectors can be introduced into mammalian host cells by standard transfection methods.

III.2.8.2. Mechanism of repression

In the absence of tetracycline, the Tet repressor forms a homodimer that binds with extremely high affinity to each $TetO_2$ sequence in the promoter of the inducible expression vector (Hillen and Berens, 1994). The 2 $TetO_2$ sites in the promoter of the inducible expression vector serve as binding sites for 4 molecules (or 2 homodimers) of the Tet repressor (Figure 6).

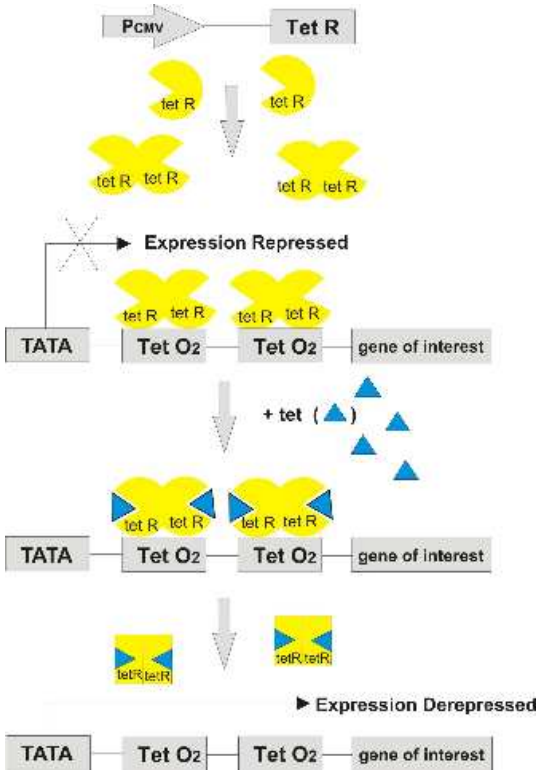


Figure 6. The components of the T-REx System.

Binding of the Tet repressor homodimers to the $TetO_2$ sequences represses transcription of gene of interest. Upon addition, tetracycline binds with high affinity to each Tet repressor homodimer in a 1:1 stoichiometry and causes a conformational change in the repressor that renders it unable to bind to the Tet operator. The Tet repressor:tetracycline complex then dissociates from the Tet operator and allows induction of transcription from the gene of interest.

III.2.8.3. Generating T-REx-293 cell lines stably expressed human SREBP-1 isoforms

The T-REx-293 cell lines, provided from Invitrogen, stably express the Tet repressor from the pcDNA6/TR plasmid and were maintained in medium containing antibiotic blasticidin (5 µg/ml). To generate stable tetracycline-inducible SREBPs cell lines, the coding regions for wild-type SREBP-1c, SREBP-1c containing the single-nucleotide polymorphism 3'322C/G and the splice variant SREBP-1d were subcloned from the pcDNA3 expression vector into pcDNA4/TO inducible expression vector (Invitrogen) using *Hind* III and *Xho* I restriction sites. The yielding plasmids were designated pcDNA4/TO-wtSREBP-1c, pcDNA4/TO-SREBP-1c-3'322C/G and pcDNA4/TO-SREBP-1d. To obtain stable transfectants, pcDNA4/TO-SREBPs constructs were linearized before transfection. Linearizing decreased the likelihood of the vector integrating into the genome in a way that disrupts the gene of interest or other elements required for mammalian expression. It is important that insert does not contain this restriction enzyme site. Suitable restriction enzymes for linearization of SREBPs plasmids was *Nru* I enzyme (New England Biolabs). Transfection was performed in dishes of 100 mm diameter according to the protocol for transient transfection with GenePorter2 Reagent (Gene Therapy Systems). About 24 hours post transfection, the medium was exchange to medium supplemented with 200 µg/ml of Zeocin. The amount of Zeocin required to kill cells not expressing the construct was titrated for T-REx-293 cell lines. The medium was exchanged for every 3-4 days. This is the time needed for the Zeocin to act on the nontransfected cells, which then detach and washed away during the medium exchange. Once all cells have died in the dish of the negative control (nontransfected), it can be proceed with the cloning.

To pick colonies 24-well dishes with 1 ml media containing 200 µg/ml Zeocin in each well were prepared. The dish was rinse with PBS and warm PBS containing 5% trypsin was added. Colonies were picked on an inverted light microscope using an Eppendorf pipette with sterile tip. The tip was lowered to the surface of colony of interest and scraped and sucked gently until the cells were pulled up into the tip. The colony was then transferred to a well in the

24-well plate. In a one or two days, when the wells were confluent, cells were trypsinized with 100 µl of trypsin-EDTA solution and splited into one well of a 6-well plate. At the level of 6-well plates, it was possible to make a first cryotube of each selected clone.

III.2.8.4. Screening of clones for SREBP-1 expression in T-REx-293 cells

The clones were screened for SREBP-1 expression by plating on 60-mm dishes at a density of 1 million cells/100mm dish and cultured in medium A (DMEM supplemented with 5% (v/v) FBS, 100 units/ml penicillin, and 100 µg/ml streptomycin) for 16 h in the presence or absence of 1 µg/ml tetracycline. After the total RNA isolation (Qiagen), cDNA was prepared (Invitrogen) and analyzed by semi-quantitative RT-PCR for induction of SREBP-1c, SREBP-1c-3'322C/G and SREBP-1d in the presence or absence of tetracycline as described above. The cell line stabily transfected with the pcDNA4/TO expression vector was used as a control for tetracycline induction of endogenous SREBP-1.

III.2.9.Quantitative PCR with TaqMan assay

III.2.9.1. Principle of TaqMan assay

The PCR reaction exploits the 5´ nuclease activity of AmpliTaq Gold DNA Polymerase to cleave a TaqMan probe during PCR. The TaqMan probe contains a reporter dye at the 5´ end of the probe and a quencher dye at the 3´ end of the probe. During the reaction, cleavage of the probe separates the reporter dye and the quencher dye, which results in increased fluorescence of the reporter. Accumulation of PCR products is detected directly by monitoring the increase in fluorescence of the reporter dye. The figure 7 shows the polymerization-associated 5´ to 3´ nuclease activity of AmpliTaq Gold enzyme during PCR. When the probe is intact, the proximity of the reporter dye to the quencher dye results in suppression of the reporter fluorescence primarily by Förster-type energy transfer (Förster, 1948; Lakowicz, 1983). During PCR, if the target of interest is present, the probe specifically anneals between the

forward and reverse primer sites. The 5´ to 3´ nucleolytic activity of the AmpliTaq Gold enzyme cleaves the probe between the reporter and the quencher only if the probe hybridizes to the target. The probe fragments are then displaced from the target, and polymerization of the strand continues.

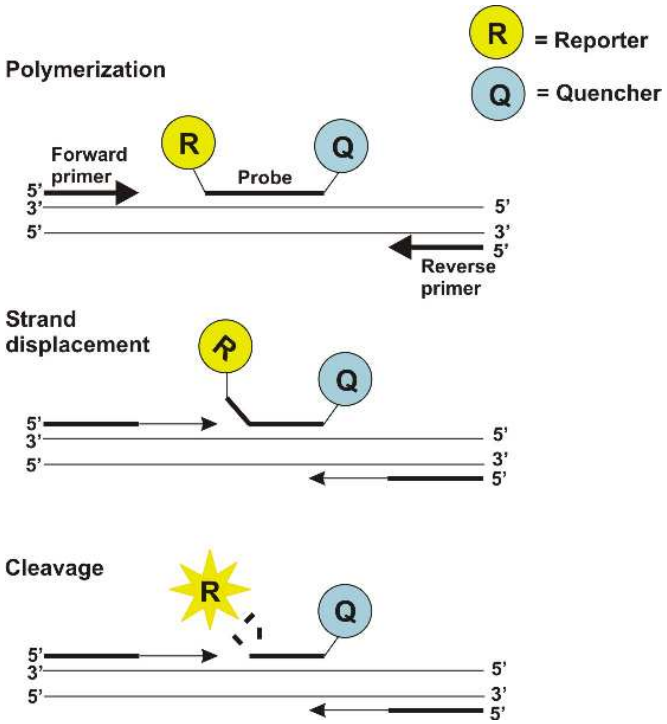


Figure 7. Schematic representation of the TaqMan assay during one extension phase of PCR.

The 3´ end of the probe is blocked to prevent extension of the probe during PCR. This process occurs in every cycle and does not interfere with the exponential accumulation of product. The increase in fluorescence signal is detected only if the target sequence is complementary to the probe and is amplified during PCR. Because of these requirements, non-specific amplification is not detected.

III.2.9.2. Setup for TaqMan assay

For TaqMan analysis of mRNA abundance, stably transfected T-Rex-293 cells were plated on 60-mm dishes at a density of 1 million cells/dish and cultured

in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum for 16 h in the presence of 1 µg/ml tetracycline. Transcription was inhibited by the addition of actinomycin D (Sigma). Actinomycin D (5 µg/ml) was added to the growth medium, and the cells were incubated for the indicated time. Total cellular RNA from stably transfected cells was isolated using Trizol reagent (Invitrogen) followed by an RNeasy total RNA isolation mini-prep system (Qiagen). cDNA was used as a template for the real-time PCR analysis based on the 5'-nuclease assay with the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, PE Europe B.V., Rotkreuz, Switzerland). PCR primers and TaqMan probe were designed by using Primer-Express software (Applied Biosystems). The probe was labeled with the fluorescent dyes 6-carboxyfluorescein (6FAM) on the 5' end and minor groove binder/non-fluorescent quencher (MGB) on the 3' end. Expression of human SREBP-1c gene was analyzed using forward primer: 3'- CGG AGC CAT GGA TTG CA-5', reverse primer: 3'- AGC ATA GGG TGG GTC AAA TAG G-5' and the probe: 6FAM-TTT CGA AGA CAT GCT TCA-MGB. The probe was placed across the exon 1c/exon 2 boundary amplifying only that transcript which corresponded to human SREBP-1c. Glyceraldehyde phosphate dehydrogenase (GAPDH) was amplified from all samples on each plate as a housekeeping gene to normalize expression between different samples and to monitor assay reproducibility. GAPDH mRNA was quantified by RT-PCR using primers and probes purchased from Applied Biosystems Assays-on-Demand #Hs99999905_m1. cDNA corresponding to 15 ng of reverse transcribed total RNA was amplified in a 25 µl volume reaction using TaqMan universal PCR Master Mix (Applied Biosystems) in triplicate assays for both SREBP-1c targets and the endogenous control GAPDH. Primers for SREBP-1c gene (Applied Biosystems) were used at a concentration of 300 nM each, FAM/MGB fluorophore/non-fluorescent quencher reporter probe (Applied Biosystems) at a concentration of 200 nM. Thermocycling conditions were as for standard TaqMan protocol (Applied Biosystems), 10 min denaturation at 95°C followed by 40 cycles of 95°C for 15 s, 60°C for 60 s. The primers and probe were validated for amplification efficiency by running the reaction at different dilutions of template and subtracting the results obtained for the control GAPDH from those of the target, this established that

the amplification efficiency was the same for all primer pairs. Relative quantification of all targets was calculated by the comparative cycle threshold (C_t) method outlined in user bulletin No. 2 provided by Applied Biosystems. The C_t is the cycle number at which the fluorescence of the sample crosses a given threshold. This threshold is set within the linear log phase of each reaction, i.e. where amplification of the product is still exponential and therefore a direct correlation can be made with the relative amounts of starting template in each reaction. The expression levels of the wild-type SREBP-1c was arbitrarily taken as the ‘calibrator' for all calculations. A non-template control was included for each target analyzed.

III.2.10.Statistical methods

RepGene, a spreadsheet template for the management of reporter gene assays was used for planning and evaluation of the experiments (Muller *et al*., 2001). The significance level of differences of means from normally distributed values was determined by one way and two way Analysis of Variance (ANOVA) using StatView 4.5 (Abacus Concepts Inc., Berkeley, CA, USA).

III. Results

III.1. The role of SREBP-1c in drug induced hyperlipidemia

III.1.1. The effects of HIV-protease inhibitor indinavir on SREBP-1c

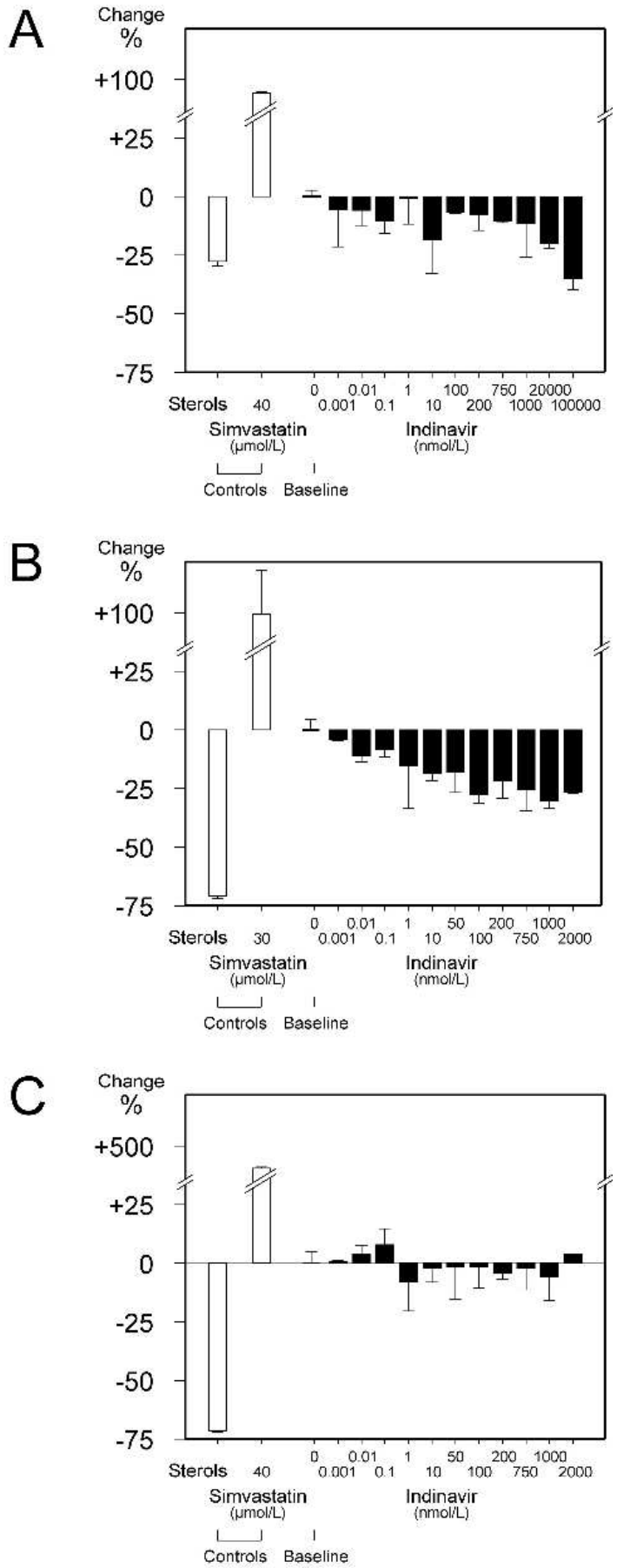
III.1.1.1. Indinavir inhibits SREBP-1c-dependent lipoprotein lipase and fatty acid synthase gene activations

As it has been outlined before, SREBP-1c is primarily involved in the regulation of the fatty acids, triglyceride and glucose metabolism, whereas SREBP-2 is mainly involved in the regulation of cholesterol. Therefore we constructed reporter genes, which allowed us to differentiate between the activation of SREBP-1c and SREBP-2, respectively.

To measure the activation of SREBP-1c, we transfected human embryonic kidney (HEK) 293 cells with a fatty acid synthase (*FAS*) and a lipoprotein lipase (*LPL*) reporter genes. To measure the activation of SREBP-2, HEK 293 cells were transfected with low-density lipoprotein receptor (*LDLR*) reporter gene. Twenty-four hours after transfection, indinavir was added at final concentrations of 0, 10^{-3}, 10^{-2},10^{-1}, 1, 10^{1}, $5x10^{1}$, 10^{2}, $2x10^{2}$, $7.5x10^{2}$, 10^{3}, $2x10^{3}$, $5x10^{3}$, $2x10^{4}$, and 10^{5} nmol/l. As a control of the inhibition of the SREBP-regulated reporter genes, the cells were incubated with 25-hydroxycholesterol (1 µg/ml) and cholesterol (10 µg/ml); as a control of the activation of the SREBP-regulated reporter genes, the cells were incubated with the dihydroxy-open form of simvastatin at final concentrations of $3x10^{4}$ or $4x10^{4}$ nmol/l.

Following incubation for 24 h, the luciferase and *Renilla* activities were determined by the Dual-Luciferase™ Reporter Assay System as described in Materials and Methods. Luciferase activities were normalized according to the *Renilla* activities.

It has been found that indinavir inhibited the *LPL* and *FAS* reporter gene activities, measured as normalized relative light units, in a dose-dependent fashion (Figure 8 A, B).


A
Change
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-50
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Sterols 40
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0.001 0.1 10 100 750 2000
Simvastatin
(µmol/L)
Indinavir
(nmol/L)
Controls Baseline

Figure 8. The effect of indinavir on the sterol-regulatory element-binding protein-1c (SREBP-1c)-dependent gene expression. (A) Effect on expression of the gene encoding the lipoprotein lipase (*LPS*). (B) Effect on expression of the gene encoding the fatty acid synthase (*FAS*). (C) Effect on expression of the gene encoding the low-density lipoprotein receptor (*LDLR*). Three independent experiments (A-C), each performed in duplicate, showing changes in the *Renilla*-normalized luciferase activities (±SEM) of the *LPL*, *FAS*, and *LDLR* reporter gene constructs. Changes are given as percentage from baseline activation of the SREBP-1c (*LPL* and *FAS*) and the SREBP-2 (*LDLR*) pathways. Sterols decrease (inhibition control, first bar in each panel) and sterol depletion increases (stimulation control, second bar in each panel) the endogenous SREBP-1c and SREBP-2 pathways, reflected by the respective reporter gene activities. The cells maintained their triglyceride and cholesterol homeostasis by a basal SREBP-1c gene activity (baseline, third bar). Indinavir inhibited *LPL* and *FAS* gene activities in a dose-dependent fashion but did not inhibit the *LDLR* gene activity.

ANOVA confirmed the significance of the interaction between the different indinavir concentrations and the inhibition of the *LPL* gene activity (P = 0.0158) as well as the interaction between the different indinavir concentrations and the inhibition of the *FAS* gene activity (P = 0.0358). Indinavir did not inhibit the *LDLR* reporter gene activity significantly.

In the *LPL* reporter gene experiments, inhibition of the gene activity was detectable starting from an indinavir concentration of 1 nmol/l. At a concentration of 10^3 nmol/l, indinavir inhibited the *LPL* gene activity from baseline by 12.4% but did not reach a plateau at this concentration. At the highest concentration tested (10^5 nmol/l) indinavir inhibited the *LPL* gene activity from baseline by 57.1% (difference baseline versus highest concentration: P = 0.041) (Figure 8A).

In the *FAS* reporter gene experiments, inhibition of the gene activity was detectable starting from an indinavir concentration of 10^{-2} nmol/l. At a concentration of 10^3 nmol/l, indinavir inhibited the *FAS* gene activity from baseline by 30.3%. The effect reached a plateau at this concentration (Figure 8B).

III.1.1.2. Reversibility of indinavir effects

To determine whether indinavir-induced inhibition of the activation of the SREBP-dependent genes was reversible or not, two sets of HEK293 cells were cotransfected with pGL2-*FAS* luciferase reporter gene constructs and with *Renilla* luciferase. These experiments were identical to those described above except that two sets of cells were used instead of one. Both sets of cells were first incubated with indinavir for 24 h at the concentrations specified above. Thereafter, indinavir was completely washed out with 1 × PBS. The first set of cells was incubated for another 24 h with fresh medium without indinavir. The second set of cells was incubated for another 24 h with fresh medium again containing indinavir at various concentrations. After incubation, the cells were harvested to determine the activation of the SREBP-dependent genes as described in Materials and Methods.

After a second incubation period, no statistically significant differences regarding the indinavir-induced inhibition of the SREBP-dependent genes were detectable between the two series. It was, therefore, concluded that indinavir-induced effects were not reversible.

III.1.1.3. Antagonization of indinavir effects by simvastatin

Toxicity of indinavir causing a decrease in the viability of the cells with increasing concentrations of indinavir was first excluded on the morphological level. Cell toxicity was not detected microscopically, even at high indinavir concentration (>10^3 nmol/l). Cell toxicity as an explanation for the decrease of the *LPL* and *FAS* gene activities attributed to indinavir was further excluded by incubating the cells with the active dihydroxy-open form of simvastatin, a strong activator of the SREBP pathways. The net effects of the combination of simvastatin (constant final concentration of 3 × 10^4 nmol/l) and indinavir (final concentrations of 10, 5 × 10^1, 10^2, 2 × 10^2 nmol/l) were determined following 24 h of incubation with the respective combinations.

When simvastatin was combined with various concentrations of indinavir, the cells were still able to upregulate the SREBP-1c-dependent genes, as shown for *FAS* (Figure 9): increasing concentrations of indinavir (0, 10, 5 × 10^1, 10^2,

and 2 × 10^2 nmol/l) decreased the rate of the gene activation induced by a constant simvastatin concentration (3 × 10^4 nmol/l) again in a dose-dependent fashion. At an indinavir concentration of 2 × 10^2 nmol/l combined with a simvastatin concentration of 3 × 10^4 nmol/l, the *FAS* gene activity decreased by 36.4 % compared with the activation achieved with simvastatin alone. However, the combination of 3 × 10^4 nmol/l simvastatin with 2 × 10^2 nmol/l indinavir still resulted in a net activation of the gene, demonstrating that the cells maintained their ability to upregulate their SREBP-1c-dependent genes despite the presence of indinavir (Figure 9).

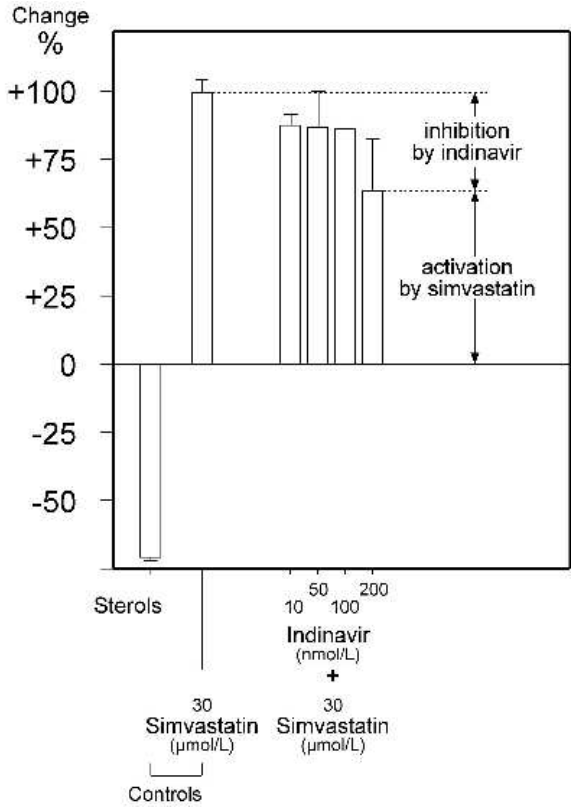

Figure 9. Antagonization of the indinavir-induced inhibition of sterol-regulatory element-binding protein (SREBP)-dependent genes by simvastatin. Experiments performed in duplicates showing changes in *Renilla*-normalized luciferase activities (±SEM) of the fatty acid synthase (*FAS*) reporter gene constructs. Changes are given as percentage from the respective baselines. If the activation by simvastatin is defined as baseline, indinavir inhibits the simvastatin-induced activation of the *FAS* gene activity in a dose-dependent fashion by 12.1% (indinavir at a final concentration of 10 nmol/l), 12.7% (5 × 10^1 nmol/l), 13.5% (10^2 nmol/l), and 36.4 % (2 x 10^2 nmol/l).

If the *FAS* gene activity without simvastatin is defined as baseline, simvastatin entirely antagonizes the indinavir-induced inhibition of the *FAS* gene activity resulting in a net activation of the *FAS* gene of 87.4% (indinavir at a final concentration of 10 nmol/l), 86.9% (5 x 10^1 nmol/l), 86.1% (10^2 nmol/l), and 63.2% (2 × 10^2 nmol/l).

The effects of indinavir on the *LPL* and *FAS* reporter genes were similarly detectable when liver cells (HepG2 cells) instead of HEK293 cells were transfected (data not shown). The effects of indinavir on the *LPL* and *FAS* reporter genes were also detectable when the cells were incubated in FCS or LPDS media. Indinavir, sterols or simvastatin did not influence the activity of the empty vectors (pGL, pRL) (data not shown).

III.1.2. The role of single-nucleotide polymorphism in the SREBP-1c gene in protease inhibitor-induced hyperlipoproteinemia

III.1.2.1. The human SREBP-1c single-nucleotide polymorphism 3'322C/G in exon 18c is not associated with alternative splicing

To investigate the molecular basis of SNP-associated differences, we first excluded the possibility that SREBP-1c-3'322G causes alternative splicing. Various human cDNA libraries and lymphocytes from several patients were screened for alternative transcripts by reverse transcriptase polymerase chain reaction using primers homologous to exon 17 and 3'-untranslated region of SREBP-1c. We did not find an association between the new discovered alternative splicing of SREBP-1 at the 3' end of mRNA and the single-nucleotide polymorphism 3'322C/G in exon 18c. The splice variants were detected in different quantities independently from 3'322C/G genotype carriers. This finding demonstrated that the 3'322C/G mutation is not associated with new discovered alternative slicing.

III.1.2.2. Putative differences of SREBP-1c mRNA secondary structures

To investigate whether the 3'322C/G SNP might cause functional differences on the mRNA level, we compared the secondary structures of the wild-type

and mutated mRNA molecules of exon 18c of the SREBP-1c gene, predicted by the MFOLD program, version 3.0 (Mathews *et al.*, 1999). This computer-based analysis discovered the conformational changes of SREBP-1c-3‘322C/G at the mRNA level. Figure 10 demonstrate the proposed differences in the predicted mRNA secondary structure.

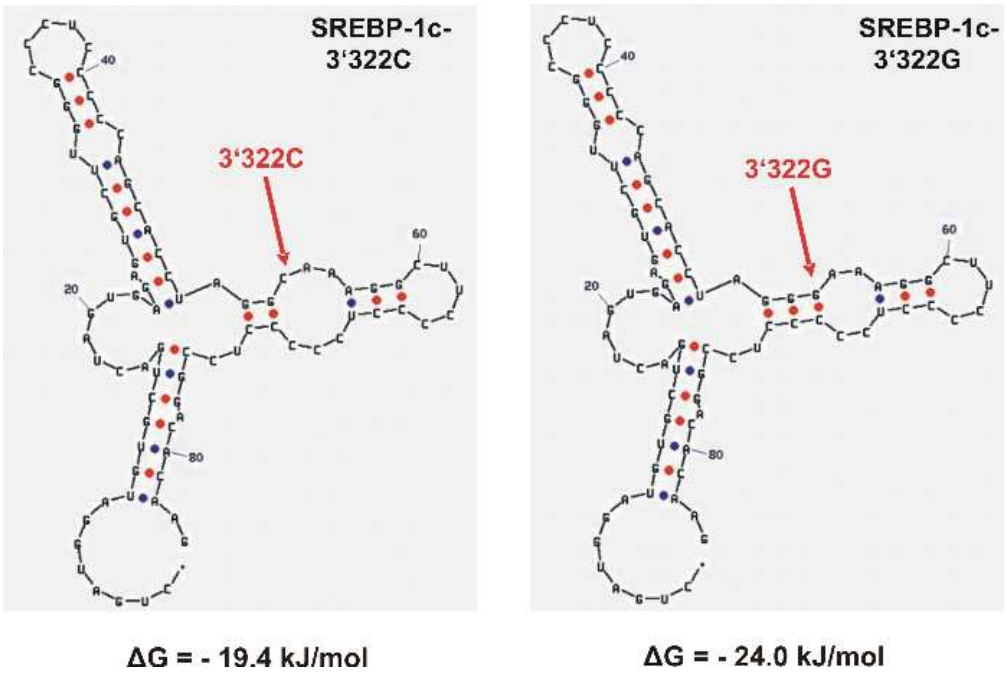

Figure 10. RNA secondary structure prediction using MFOLD 3.0 (Mathews *et al.*, 1999).

As shown in Figure 10, both SREBP-1c mRNAs have different free energy. Wild-type mRNA (C isoform, genotype 11) of SREBP-1c has extended single-nucleotide stem loop. Whereas mutated mRNA (G isoform, genotype 22) of SREBP-1c has an additional nucleotide duplex which increase the stability of this isoform. This finding is especially important because we suppose that the mRNA stability of the SREBP-1c gene seems to be crucial for its regulation in highly active antiretroviral treatment.

III.1.2.3. Establishment of the stable T-REx-293 cell lines permitting inducible expression of wild-type SREBP-1c and the SREBP-1c containing the single-nucleotide polymorphism 3'322C/G

An expression plasmid for human wild-type SREBP-1c was constructed by cloning full-length coding sequences and the entire 3' untranslated region (3' UTR) including a polyadenylation signal (AATAAA) of SREBP-1c from HeLa cDNA library into the pcDNA3 expression vector. To obtain a fragment containing the sequence variant in exon 18c (3'322C/G), *in vitro* mutagenesis was applied to part of the exon 18c of the SREBP-1c gene. For the mRNA stability experiments two different full-length cDNAs, the wild-type SREBP-1c and the SREBP-1c containing the single-nucleotide polymorphism 3'322C/G (SREBP-1c-3'322C/G), were compared. The T-REx inducible system to achieve tightly controlled and conditional expression of the SREBP-1c isoforms in 293 cells was employed. For that both full-length cDNA inserts (SREBP-1c-3'322C and SREBP-1c-3'322G) were cloned into the multiple cloning site of the inducible tetracycline-regulated expression (T-REx) vector pcDNA4/TO (Invitrogen).

T-REx-293 cells, which stably express the tetracycline (Tet) repressor, were transfected with expression vector pcDNA4/TO, carrying the wild-type SREBP-1c or SREBP-1c-3'322C/G. Two-round stable transfection experiments were performed, and several zeocin-resistant clones selected. The clones were screened for the wild-type SREBP-1c and SREBP-1c-3'322C/G expression by culturing the cells in medium in the presence or absence of tetracycline (1 µg/ml). RNA and cDNA were prepared and analyzed by semi-quantitative RT-PCR for induction of SREBP-1c and SREBP-1c-3'322C/G with tetracycline as described in Materials and Methods. The cell line stably transfected with pcDNA4/TO vector was used as a control for tetracycline induction of endogenous SREBP-1c. After screening by semi-quantitative RT-PCR for induction of SREBPs, we established zeocin-resistant clones which positively express the transgenes. These clones, termed wt-SREBP-1c and SREBP-1c-3'322C/G, which actually represented almost undetectable background expression under non-induced conditions, show remarkable expression of different SREBPs mRNA after tetracycline

induction (Figure 11). These stable cell line (wt-SREBP-1c and SREBP-1c-3'322C/G) were used as a tool to study the influence of SREBP-1c SNP 3'322C/G on mRNA stability and explain disorders in lipid metabolism in HIV-1-infected patients after PIs treatment.

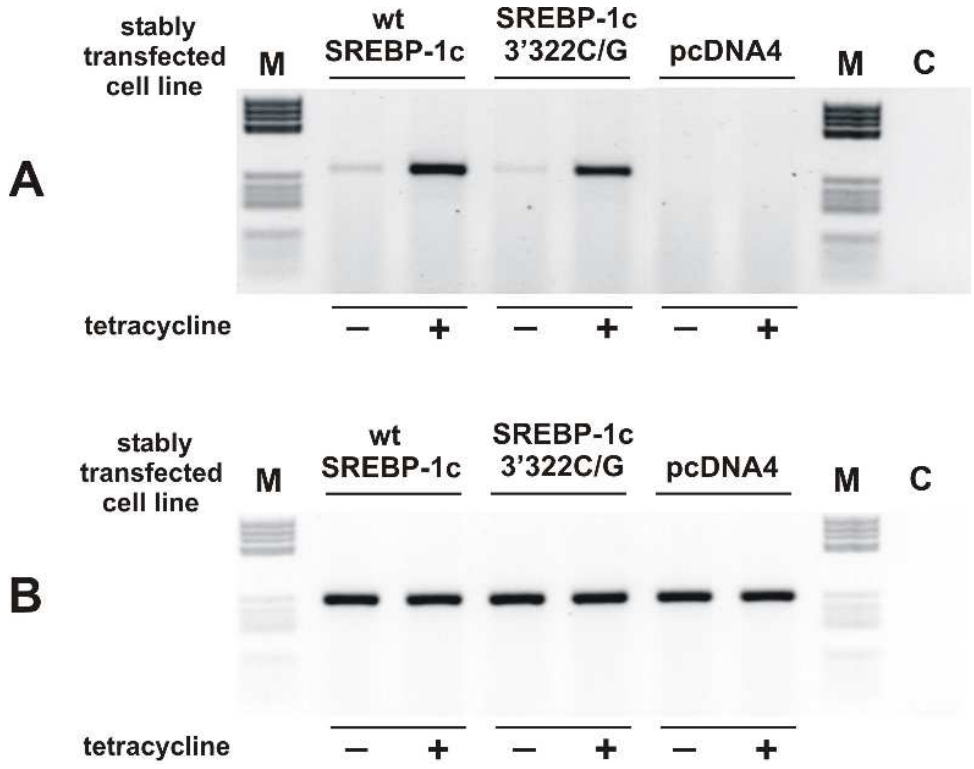

Figure 11. The tetracycline induce stably transfected wild-type SREBP-1c and the SREBP-1c-3'322C/G in T-REx-293 cells. T-REx-293 cells stably transfected with indicated expression constructs (wild-type SREBP-1c and SREBP-1c-3'322C/G) were treated for 16 hours with tetracycline (1 µg/ml). The cell line stably transfected with pcDNA4/TO vector was used as a control for tetracycline induction of endogenous SREBP-1c. Total RNA (5 µg) was used to prepare cDNA by reverse transcription with random hexamers primers followed by the semi-quantitative RT-PCR using primers homologous to exon 17 and 3'-untranslated region of SREBP-1c (F ex17 and R 3'UTR primers, respectively) (**A**) and the internal control GAPDH (**B**) as indicated. PCR products were resolved by electrophoresis on a 2% agarose gel containing ethidium bromide. M, DNA molecular weight marker V (Roche Diagnostics AG, Rotkreuz, Switzerland). C, control (no template).

III.1.2.4. Characterization of wild-type SREBP-1c and the SREBP-1c-3'322C/G stable transformants by transient transfection of luciferase reporter constructs

Two luciferase reporter constructs containing the promoter region of fatty acid synthase gene (pGL2-*FAS*) and lipoprotein lipase gene (pGL2-*LPL*) were used for characterization of SREBP-1 stable transformants in T-REx-293 cells. Each luciferase reporter construct was transfected into T-REx-293 cell lines stably expressed either with wild-type SREBP-1c or the SREBP-1c containing the SNP 3'322C/G (SREBP-1c-3'322C/G). After transfection, the cells were incubated with tetracycline (1 µg/ml) for induction of wild-type SREBP-1c and SREBP-1c-3'322C/G isoforms. The cell line, stably transfected with the pcDNA4/TO vector only, was used as a control for tetracycline induction of endogenous SREBP-1c. After induction of SREBPs, cells were treated with 25-hydroxycholesterol and cholesterol as a control of the inhibition of the SREBP-regulated reporter genes; and with the dihydroxy-open form of simvastatin as a control of the activation of the SREBP-regulated reporter genes.

Figure 12 compares the ability of wild-type SREBP-1c and the SREBP-1c-3'322C/G to induce transcription of the *FAS* and *LPL* luciferase reporter genes. Both, wild-type SREBP-1c and SREBP-1c-3'322C/G, transformants exhibit relatively strong transactivation of the transiently transfected reporters constructs in comparison with a control cell line, stably transfected with the pcDNA4/TO expression vector. As shown in Figure 12, the SREBP-1c-3'322C/G produced a transcriptional response that was significantly larger than the wild-type SREBP-1c when simvastatin, a SREBPs agonist, was added to the culture medium. The SREBP-1c-3'322C/G isoform activated the *FAS* and *LPL* reporter genes greater (1.6-fold, $P = 0.007$ and 2.7-fold, $P = 0.0007$, respectively) than wild-type SREBP-1c isoform.

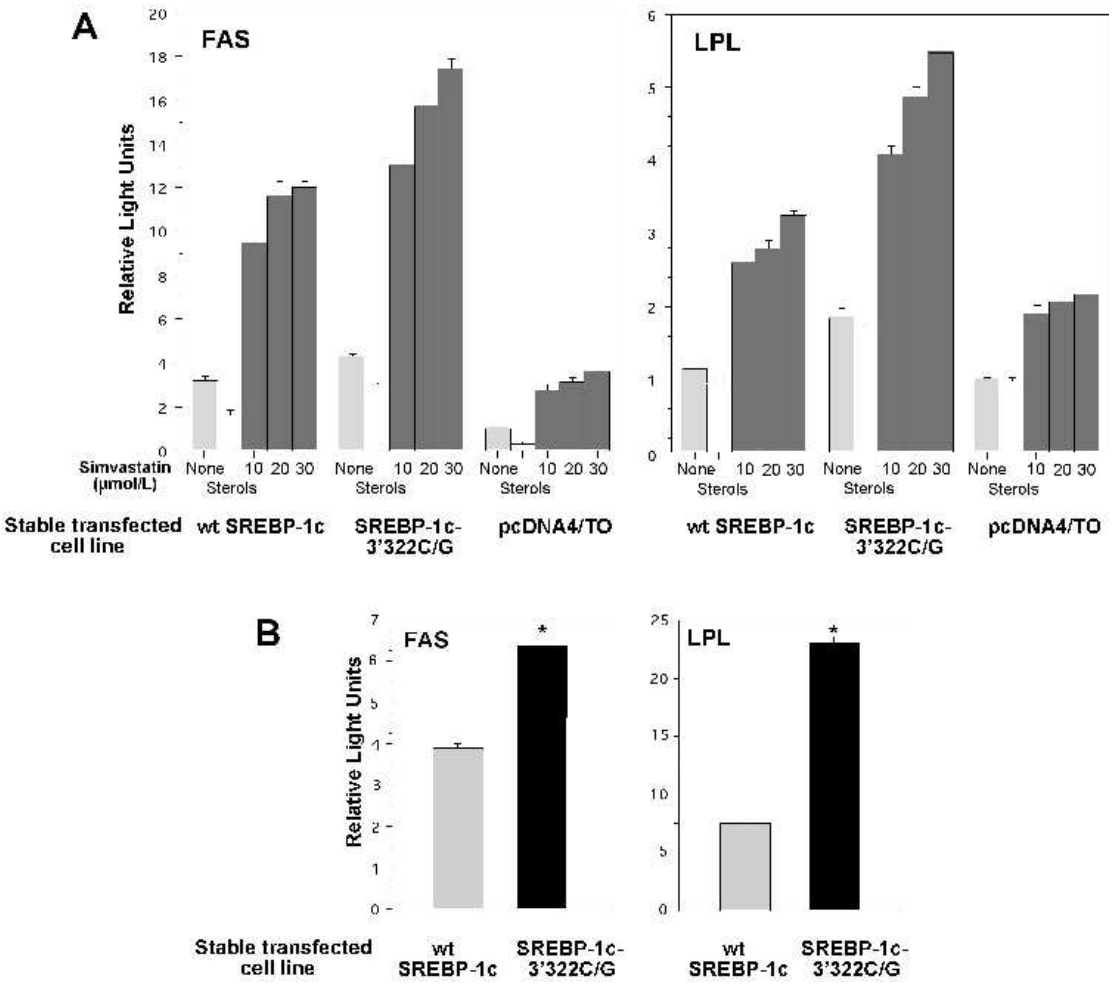

Figure 12. Characterization of SREBP-1 stable transformants of T-REx-293 cells by transient transfection of luciferase reporter constructs. The luciferase reporter constructs containing the promoter region of fatty acid synthase (pGL2-*FAS*) and lipoprotein lipase (pGL2-*LPL*) were co-transfected with the pRL-CMV vector containing *Renilla* luciferase into T-REx cell lines, stably expressing either wild-type SREBP-1c or the SREBP-1c containing SNP 3'322C/G (SREBP-1c-3'322C/G). The cell line, stably transfected with the pcDNA4/TO vector, was used as a control for tetracycline induction of endogenous SREBP-1c. Cells were than treated with 1 µg/ml 25-hydroxycholesterol and 10 µg/ml cholesterol (Sigma) as a control of the inhibition of the SREBP-regulated reporter genes; and with the dihydroxy-open form of simvastatin at final concentrations of $1x10^4$, $2x10^4$ and $3x10^4$ nmol/l as a control of the activation of the SREBP-regulated reporter genes. For all transfections, activation of luciferase was corrected for transfection efficiency with *Renilla* luciferase activity levels, and all data are represented accordingly as relative light units. **A**. Luciferase activity is expressed as the -fold increase relative to untreated (without simvastatin)

T-REx cells stably transfected with the pcDNA4/TO vector. **B**. Comparison of maximum activation of *FAS* and *LPL* reporter genes by simvastatin (30 µM) in T-REx cells stably expressing either wild-type SREBP-1c (grey) or SREBP-1c-3'322C/G (black). Luciferase activity of control T-REx cell line was subtracted to luciferase activity of cell lines stably transfected with wild-type SREBP-1c and SREBP-1c-3'322C/G. The *error bars* indicate S.D. from the mean. *, P = 0.007 (comparison between wild-type SREBP-1c and SREBP-1c-3'322C/G for *FAS* gene); P = 0.0007 (comparison between wild-type SREBP-1c and SREBP-1c-3'322C/G for *LPL*).

III.1.2.6. The influence of the SNP 3'322C/G in the SREBP-1c gene on mRNA stability

For analysis of mRNA abundance, T-REx-293 cell lines, stably expressing either wild-type SREBP-1c or the SREBP-1c containing SNP 3'322C/G (SREBP-1c-3'322C/G) were cultured in the presence of tetracycline for the induction of these isoforms. Transcription was then inhibited by the addition of actinomycin D. Actinomycin D was added to the growth medium (5 µg/ml), and the cells were incubated for the indicated time (Figure 13).

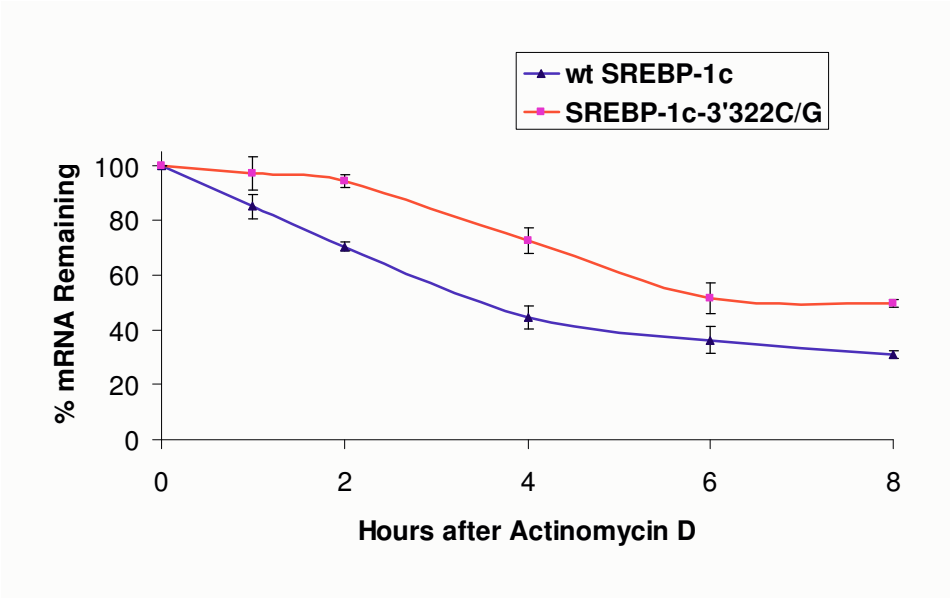

Figure 13. The influence of the SNP 3'322C/G in the SREBP-1c gene on mRNA stability. T-REx-293 cells stably expressing wild-type SREBP-1c and the SREBP-1c-3'322C/G, were treated for 16 hours with tetracycline (1 µg/ml). Actinomycin D than was added to 5 µg/ml, and total cellular RNA was harvested after an additional 1, 2, 4, 6 or 8 hours. SREBP-1c mRNA levels were evaluated with a real time quantitative

polymerase chain reaction method as described in Materials and Methods. The *error bars* indicate S.D. from the mean. The *graph* depicts the % mRNA remaining after 8 h of actinomycin D.

Total cellular RNA from stably transfected cells was isolated. cDNA was used as a template for the quantitative polymerase chain reaction analysis based on the 5'-nuclease assay with the ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Figure 13 demonstrates that mRNA of the SREBP-1c with 3'322C/G polymorphism is more stable than wild-type SREBP-1c mRNA (P = 0.02). SREBP-1c-3'322C/G mRNA has a half-life of about 6 hours, whereas wild-type SREBP-1c has a half-life of about 3.5 hours. These data suggest that sequence differences in the coding 3'-end of the SREBP-1c gene (3'322C/G SNP) affects the secondary structure of the SREBP-1c mRNA and influence its degradation rate.

III.2. Alternative splicing of human SREBP-1

III.2.1. Identification and characterization of new splice variants of human SREBP-1

In the process of cloning of the SREBP-1c gene for further studies we observed a number of different mRNA transcripts indicating possible alternative splicing of this gene in humans at the 3' end of pre-mRNA. We analyzed these transcripts using reverse transcriptase polymerase chain reaction (RT-PCR) experiments in lymphocytes from several individuals. To verify the identity of the PCR products, the bands were excised and subjected to DNA sequencing. RT-PCR was conducted using SREBP-1c-specific primers that span exon 17 and 3'-untranslated region. First, we identified new splice variants in human liver and HeLa cells cDNA libraries. Sequencing of these transcripts revealed that they contain various combinations of the a and c exons at the 3' end. For example, liver cDNA mRNAs contain only SREBP-1 transcript with exons 17, 18a and 18c. It is interesting, that this library does not contain wild-type SREBP-1c transcripts. In contrast, HeLa cells cDNA library contains wild-type SREBP-1c (with exons 17, 18c and 19c) and

another splice variant with exons 17, 18a, 18c and 19c. Second, we analyzed the splice variants that are common in human lymphocytes. Total RNA was isolated from lymphocytes of various (n=95) individuals, cDNA was prepared and subjected to RT-PCR. During this process a third splice variant was detected and identified by sequence analysis. This new splice variant contained exons 17 and 18c, but exon 19c and a part of the 3'-untranslated region were spliced out.

We tentatively designated the human SREBP-1 splice variant containing exons 17, 18a and 18c as SREBP-1d. Splice variant containing exons 17, 18a, 18c and 19c as SREBP-1e, and splice variant containing exons 17 and 18c as SREBP-1f (Figure 14).

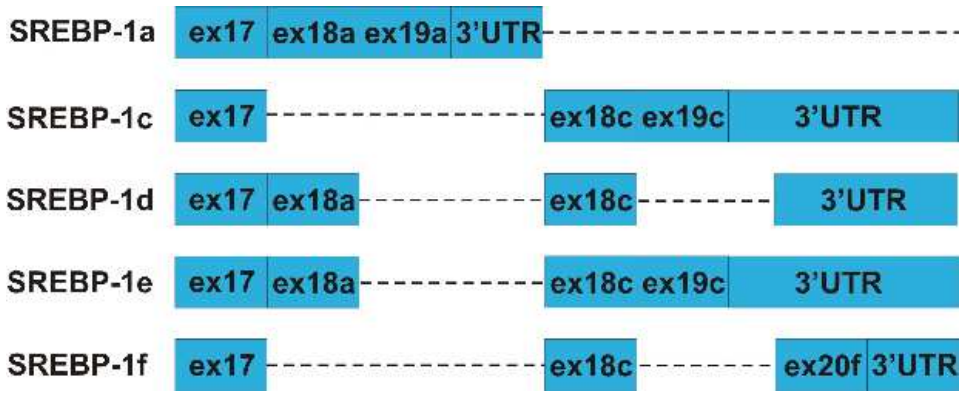

Figure 14. Splice variants of human SREBP-1 at the 3' end.

Interestingly, the lymphocytes of the numerous individuals investigated had in the most cases (62%) all splice variants at different expression levels (Table 1). About 13% of them expressed all splice variants except the SREBP-1d isoform. Furthermore, the 8% lymphocytes expressed all splice variants without wild-type SREBP-1c isoform. The 6% and 5% lymphocytes expressed only SREBP-1d and SREBP-1c isoforms or SREBP-1d and SREBP-1f isoforms, respectively. The frequency of other combinations of the SREBP-1 splice variants were more less (1%).

SREBP-1e	SREBP-1d	SREBP-1c	SREBP-1c	SREBP-1 isoforms in lymphocytes, %
+	+	+	+	62
	+	+	+	13
+	+		+	8
	+		+	6
	+	+		5
+		+		1
	+			1
+		+	+	1
+	+	+		1
+	+			1

Table 1. The frequency (%) of the SREBP-1 splice variants in human lymphocytes of numerous (n=95) individuals. Total RNA (5 µg) from lymphocytes were used to prepare cDNA by reverse transcription with random hexamers primers. PCR amplification was carried out using primers specific for SREBP-1c exon 17 and 3'UTR (F ex17 and R 3'UTR primers, respectively). PCR products were analyzed by electrophoresis on a 2% agarose gel, and band intensities were compared by imaging of ethidium bromide staining. A plus sign denotes presence of certain splice variant.

The results of the RT-PCR of the cDNA obtained from lymphocytes of some of the individuals (n=4) are illustrated in Figure 15. As a control to compare and identify the new splice variants we used liver and HeLa cDNA libraries. In agreement with the results described above the individuals had a considerable variety of splice variants in different quantities.

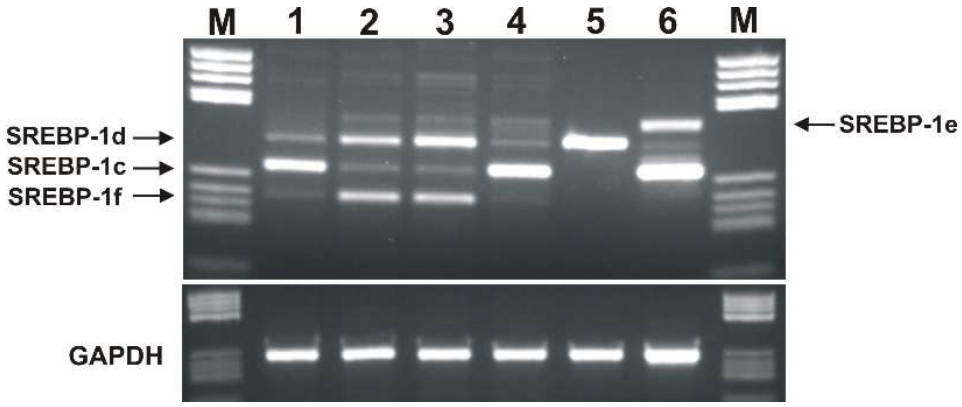

Figure 15. Identification of new splice variants of human SREBP-1 in lymphocytes mRNA. Total RNA (5 µg) from lymphocytes were used to prepare cDNA by reverse

transcription using random hexamers primers followed by RT-PCR using primers homologous to exon 17 and 3'-untranslated region of SREBP-1c (F ex17 primer and R 3'UTR primer, respectively). PCR products were resolved by electrophoresis on a 2% agarose gel containing ethidium bromide. Lymphocytes cDNA of some (n=4) patients (lanes 1-4), liver cDNA library (lane 5), HeLa cDNA library (lane 6). SREBP-1e splice variant containing exons: 17, 18a, 18c and 19c (392 bp); SREBP-1d splice variant containing exons: 17, 18a and 18c (338 bp); SREBP-1c splice variant (wild-type) containing exons: 17, 18c and 19c (280 bp); SREBP-1f splice variant containing exons: 17, 18c and 20f (226 bp). M, DNA molecular weight marker V (Roche Diagnostics).

Comparison of cDNA sequence of each transcript with the human SREBP-1a (NM004176) and SREBP-1c (AH004383) sequence from database revealed that exon 18a of human SREBP-1a gene terminates with an incomplete codon and exon 18c begins with a complete codon. This leads to a resumption of an out-of-frame code and two premature termination codons (6 amino acids after the splice site) resulting in a predicted truncated protein of SREBP-1d.

The splice variant SREBP-1e is very similar to variant SREBP-1d except that after exons 18a and 18c follows exon 19c. In a similar way after exon 18a comes the frame shift and consequently a premature termination of the coding sequence.

In splice variant SREBP-1f exon 19c with the termination codon and a part of 3' untranslated region is spliced out. The remaining part of the 3' untranslated region sequences has a possible criptic site for alternative splicing. Like all of the introns it has AG nucleotides at their 3' splice acceptor sites (aagGAG). As a result of this splicing event the new exon termed 20f (51 amino acids after the splice site followed by the termination codon) is created (Figure 14).

III.2.2. Different ways of expression the SREBP-1 isoforms

To further investigate the presence of alternatively spliced SREBP-1 mRNA in human normal and tumor tissues, multiple-tissue cDNA panels (Clontech Laboratories, Palo Alto, CA, USA) were subjected to PCR analysis using a

pair of SREBP-1c-specific primers. A pair of GAPDH-specific primers served as internal control. This analysis detected alternatively spliced SREBP-1e transcript in all normal tissues and tumor-derived cells at different detectable levels (Figure 16).

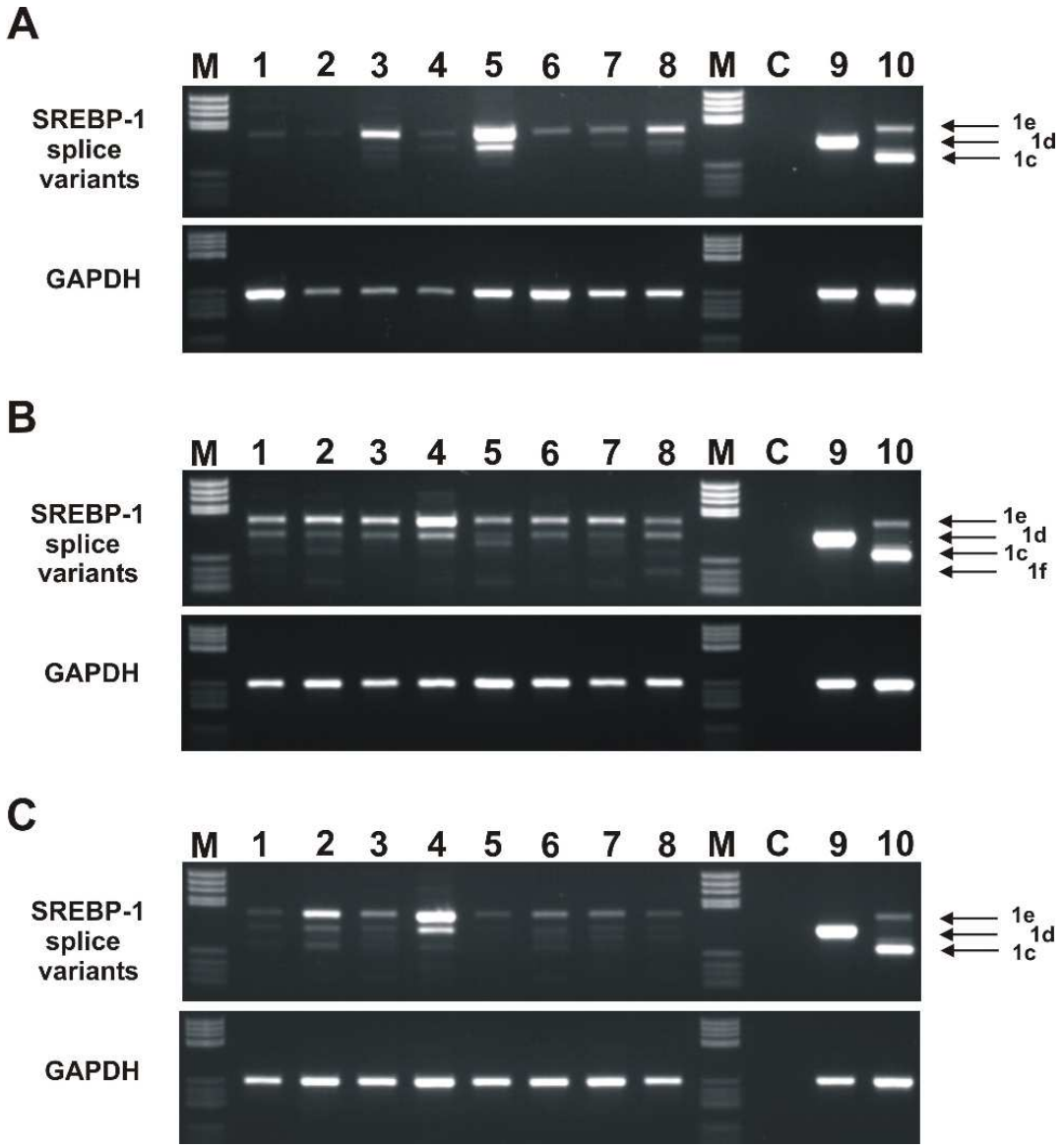

Figure 16. The mRNA level of SREBP-1 splice variants in human normal and tumor tissues. PCR amplification was carried out on panels of cDNAs from human normal tissues (**A** and **B**) and tumor tissues (**C**) using the SREBP-1c-specific primers spanning the exon 17 and 3'-untranslated region (F ex17 primer and R 3'UTR primer, respectively). The upper panels in each set show 392 bp, 338 bp, 280 bp and 226 bp DNA fragments amplified for SREBP-1e (39 cycles), SREBP-1d (39 cycles), SREBP-1c (39 cycles) and SREBP-1f (39 cycles), respectively, in a agarose gel

electrophoresis. The lower panels in each set exhibit 261 bp cDNA fragments amplified for human glyceraldehydes phosphate dehydrogenase (GAPDH, 25 cycles). C, control (no template). M, DNA molecular weight marker V (Roche Diagnostics). **A**, lane 1, heart; lane 2, brain; lane 3, placenta; lane 4, lung; lane 5, liver; lane 6, skeletal muscle; lane 7, kidney; lane 8, pancreas. **B**, lane 1,spleen; lane 2, thymus; lane 3, prostate; lane 4, testis; lane 5, ovary; lane 6, small intestine; lane 7, colon; lane 8, peripheral blood leukocyte. **C**, lane 1, breast carcinoma (GI-101); lane 2, lung carcinoma (LX-1); lane 3, colon adenocarcinoma (CX-1); lane 4, lung carcinoma (GI-117); lane 5, prostatic adenocarcinoma PC3); lane 6, colon adenocarcinoma (GI-112); lane 7, ovarian carcinoma (GI-102); lane 8, pancreatic adenocarcinoma (GI-103).

cDNA Panel I

	1 heart	2 brain	3 placenta	4 lung	5 liver	6 skeletal muscle	7 kidney	8 pancreas
SREBP-1e	±	±	+	±	+	+	+	+
SREBP-1d			±	±	+		±	±
SREBP-1c			±		±			
SREBP-1f								

cDNA Panel II

	1 spleen	2 thymus	3 prostate	4 testis	5 ovary	6 small intestine	7 colon	8 perif. blood leukocytes
SREBP-1e	+	+	+	+	+	+	+	+
SREBP-1d	+	+	+	+	+	+	±	+
SREBP-1c	±	±						±
SREBP-1f								±

Tumor cDNA Panel

	1 breast carcin. GI-101	2 lung carcin. LX-1	3 colon adenocarc. CX-1	4 lung carcin. GI-117	5 prostatic adenocarcin. PC3	6 colon adenocarcin. GI-112	7 ovarian carcin. GI-102	8 pancreatic adenocarin. GI-103
SREBP-1e	±	+	+	+	±	+	+	±
SREBP-1d	±	+	±	+		±	±	±
SREBP-1c	±	+	±	±		±	±	
SREBP-1f								

Table 2. The mRNA level of SREBP-1 splice variants in human normal and tumor tissues. PCR products were analyzed by electrophoresis on a 2% agarose gel, and band intensities were compared by imaging of ethidium bromide staining. A plus sign denotes high expression level of certain splice variant. A plus/minus sign denotes low expression level of certain splice variant.

The highest expression level was detected in liver, placenta, pancreas, testis, thymus and prostate tissues, and in lung carcinoma (LX-1 and GI-117), colon adenocarcinoma (CX-1 and GI-112) and ovarian carcinoma (GI-102) in the case of the tumor-derived cells (Table 2). The SREBP-1d transcript was detected in most of normal tissues and also in tumor-derived cells examined at different detectable levels. The highest expression was in liver and testis, and in lung carcinoma (LX-1 and GI-117) in the tumor-derived cells. On the other hand, the SREBP-1f splice variant was detected only in peripheral blood leukocyte on a exceptionally low expression level. Furthermore, the analysis showed wild-type SREBP-1c transcript at low expression level in placenta, liver, spleen, thymus and peripheral blood leukocyte and in all tumor-derived cells except prostatic and pancreatic adenocarcinoma (PC3 and GI-103, respectively). In contrast to SREBP-1e, the expression of SREBP-1d, SREBP-1c and SREBP-1f in tissues and tumor-derived cells appeared to be relatively low.

To summarize our results, the distribution pattern of human SREBP-1 splice variants is different in normal tissues and tumor-derived cells. We therefore assumed that the expression of these isoforms is regulated by distinct mechanisms.

III.2.3. Different ways of induction the SREBP-1 isoforms in HepG2 cells

Recently it has been reported that several compounds (for example glucocorticoids) can change the expression level of the splice variants of the diacylglycerol kinase (Klauck *et al.*, 1996; Murakami *et al.*, 2003). Based on these findings we decided to investigate the possible induction of certain drugs, which affect SREBP-1c expression, on the expression pattern of SREBP-1 splice variants. HepG2 cells were selected for these experiments,

because these cells retain many properties of the hepatocytes from which they are derived (Knowles *et al*., 1980). Simvastatin, a HMG-CoA reductase inhibitor and SREBP pathway activator (Brown and Goldstein, 1997), was used in this study. Additionally, the HIV-protease inhibitor indinavir, which is associated with decreased SREBP-1c expression (Miserez *et al*., 2002a) and finally, insulin, a potent inductor of SREBP-1c gene expression (Foretz *et al*., 1999; Shimomura *et al*.,1999) were tested.

HepG2 cells were grown in medium supplemented with 10% FBS. The cells were treated with 30 µM active dihydroxy-open form of simvastatin, 20 µM indinavir and 1 µM insulin for 24 hours. For conditions with low lipid concentration, the cells were maintained in medium containing 5% LPDS. After RT-PCR using the SREBP-1c-specific primers spanning the exon 17 and 3'-untranslated region, we found that all splice variants of SREBP-1 could be detected at different expression levels (Figure 17).

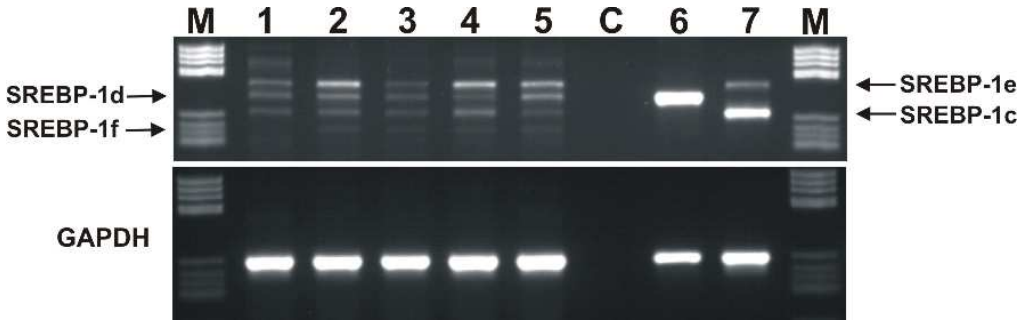

Figure 17. Expression regulation of the SREBP-1 splice variants by simvastatin, indinavir, insulin and LPDS medium. 1, HepG2 cells were maintained in 10% FCS medium and served as a control. 2, HepG2 cells were maintained in 5% LPDS medium. 3, HepG2 cells were treated with 30 µM of simvastatin. 4, HepG2 cells were treated with 20 µM indinavir. 5, HepG2 cells were treated with 1 µM insulin. The cells were incubated for 24 h. Total RNA was isolated and the aliquots (5 µg) were subjected to RT-PCR using the SREBP-1c-specific primers spanning the exon 17 and 3'-untranslated region (F ex17 primer and R 3'UTR primer, respectively). An upper panel shows 392 bp, 338 bp, 280 bp and 226 bp DNA fragments amplified for SREBP-1e (35 cycles), SREBP-1d (35 cycles), SREBP-1c (35 cycles) and SREBP-1f (35 cycles), respectively, in agarose gel electrophoresis. The lower panels exhibit 261 bp cDNA fragments amplified for human glyceraldehyde phosphate

dehydrogenase (GAPDH, 25 cycles). C, control (no template). M, DNA molecular weight marker V (Roche Diagnostics).

When HepG2 cells were grown in conditions with low lipid concentration (LPDS medium), the level of SREBP-1e mRNA was markedly increased (Figure 17). When HepG2 cells were treated with 20 µM of indinavir for 24 h, the level of SREBP-1e mRNA was also increased. In contrast, the SREBP-1d mRNA was moderately decreased. The splice variants SREBP-1d and SREBP-1e were induced by treating cells with 1 µM insulin. Interestingly, when HepG2 cells were treated with simvastatin (30 µM), the mRNA level of all SREBP-1 variants was markedly decreased (Figure 17). The mRNA level of SREBP-1c and SREBP-1f were altered weaker by changing of conditions or adding substances.
The results collectively demonstrated that the expression pattern of mRNA of SREBP-1 splice variants were clearly different from each other after addition of certain drugs (simvastatin, indinavir, insulin), that affect the SREBP-1c expression, or changing the lipid concentration (medium with low lipid level).

III.2.4. Establishment of the stable T-REx-293 cell lines permitting inducible expression of wild-type SREBP-1c and the splice variant SREBP-1d

As it has been outlined before both, SREBP-1a and -1c, isoforms are derived from a single gene through the use of alternative splicing at both, the 5' and the 3', ends of the mRNA. Exons 1a and 1c are transcribed from different promoters using two alternative transcription start sites and spliced to a common exon 2 (Figure 4). The new splice variant SREBP-1d was detected in the liver cDNA library. This isoform contained exons 17, 18a and 18c at the 3'-end of mRNA. To investigate the 5'-end of this transcript the polymerase chain reaction was performed using two forward primers homologous to exon 1a and exon 1c, and reverse primer homologous to exon 2 which is specific for SREBP-1a and SREBP-1c. This analysis revealed that the amplification occurs only when the forward primer, homologous to exon 1c of SREBP-1c isoform, was used. Therefore, the splice variant SREBP-1d contains at the 5'-

end the exon 1c, and the N-terminus of this isoform is similar to the SREBP-1c. This finding is particularly important because it has been demonstrated that the SREBP-1c (not SREBP-1a) predominate in the liver and most other intact tissues (Shimomura *et al.*, 1997a). Thus, the 3'-end of coding sequences with the entire untranslated region of SREBP-1 from human liver cDNA library was cloned into expression plasmid pcDNA4/TO-SREBP-1c yielding the expression plasmid pcDNA4/TO-SREBP-1d.

The T-REx-293 cells, which stably express the tetracycline (Tet) repressor, were transfected with the expression vector, pcDNA4/TO, carrying the wild-type SREBP-1c or the splice variant SREBP-1d. Two-round stable transfection experiments were performed, and several zeocin-resistant clones selected. The clones were screened for the SREBP-1 expression by culturing the cells in medium in the presence or absence of tetracycline (1 µg/ml). RNA and cDNA were prepared and analyzed by semi-quantitative RT-PCR for induction of SREBP-1c and SREBP-1d expression as described in Materials and Methods. The cell line stably transfected with the pcDNA4/TO vector was used as control for tetracycline induction of SREBP-1. After screening by semi-quantitative RT-PCR for induction of SREBPs we established zeocin-resistant clones which positively express the transgenes (Figure 18).

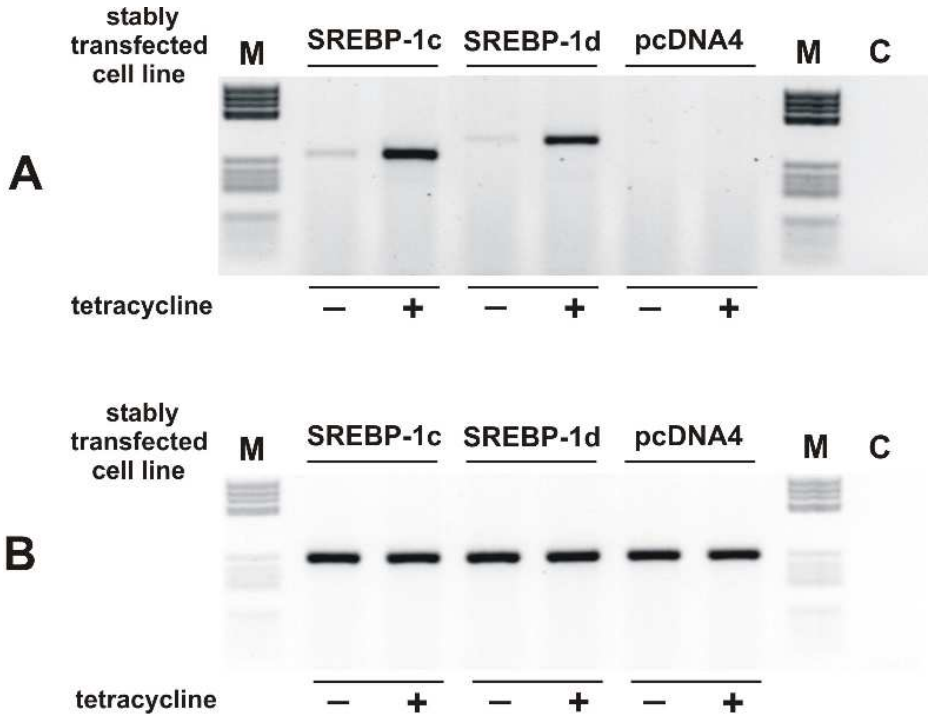

Figure 18. Induction of wild-type SREBP-1c and the splice variant SREBP-1d after tetracycline treatment in T-REx-293 cells. T-REx-293 cells, stably transfected with the indicated expression constructs, were treated for 16 hours with tetracycline (1 µg/ml). The cell line stably transfected with the pcDNA4/TO vector was used as a control for tetracycline induction of SREBP-1. Total RNA (5 µg) was used to prepare cDNA by reverse transcription with random hexamers primers followed by a semi-quantitative RT-PCR using the primers specific for SREBP-1c exon 17 and 3'UTR (**A**) and the internal control GAPDH (**B**) as indicated. PCR products were resolved by electrophoresis on a 2% agarose gel containing ethidium bromide. M, DNA molecular weight marker V (Roche Diagnostics). C, control (no template).

These clones, termed SREBP-1c and SREBP-1d, which actually represented almost undetectable background expression under non-induced conditions, showed remarkable expression of SREBPs mRNA after tetracycline induction (Figure 18).

III.2.5. Characterization of splice variant SREBP-1d in stably transfected T-REx-293 cells by transient transfection of luciferase reporter constructs

Two luciferase reporter constructs containing the promoter region of fatty acid synthase gene (pGL2-*FAS*) and lipoprotein lipase gene (pGL2-*LPL*) were used for characterization of the SREBP-1 stable transformants in T-REx-293 cells. Each luciferase reporter construct was transfected into T-REx-293 cell lines stably expressing either wild-type SREBP-1c or the splice variant SREBP-1d. After transfection, the cells were incubated with tetracycline (1 µg/ml) for induction of wild-type SREBP-1c and the splice variant SREBP-1d. The cell line stably transfected only with pcDNA4/TO vector was used as control for tetracycline induction of endogenous SREBP-1c. After induction of SREBPs, cells were treated with 25-hydroxycholesterol and cholesterol as a control of the inhibition of the SREBP-regulated reporter genes; and with the dihydroxy-open form of simvastatin as a control of the activation of the SREBP-regulated reporter genes.

Figure 19 compares the ability of wild-type SREBP-1c and the splice variant SREBP-1d to induce transcription of the *FAS* and *LPL* luciferase reporter genes. Both, wild-type SREBP-1c and the splice variant SREBP-1d, transformants exhibit relatively strong transactivation of the transiently transfected reporters constructs in comparison with a control cell line stably transfected with the pcDNA4/TO expression vector. As shown in Figure 19, wild-type SREBP-1c produced a transcriptional response that was significantly larger than the splice variant SREBP-1d when simvastatin, a SREBPs agonist, was added to the culture medium.

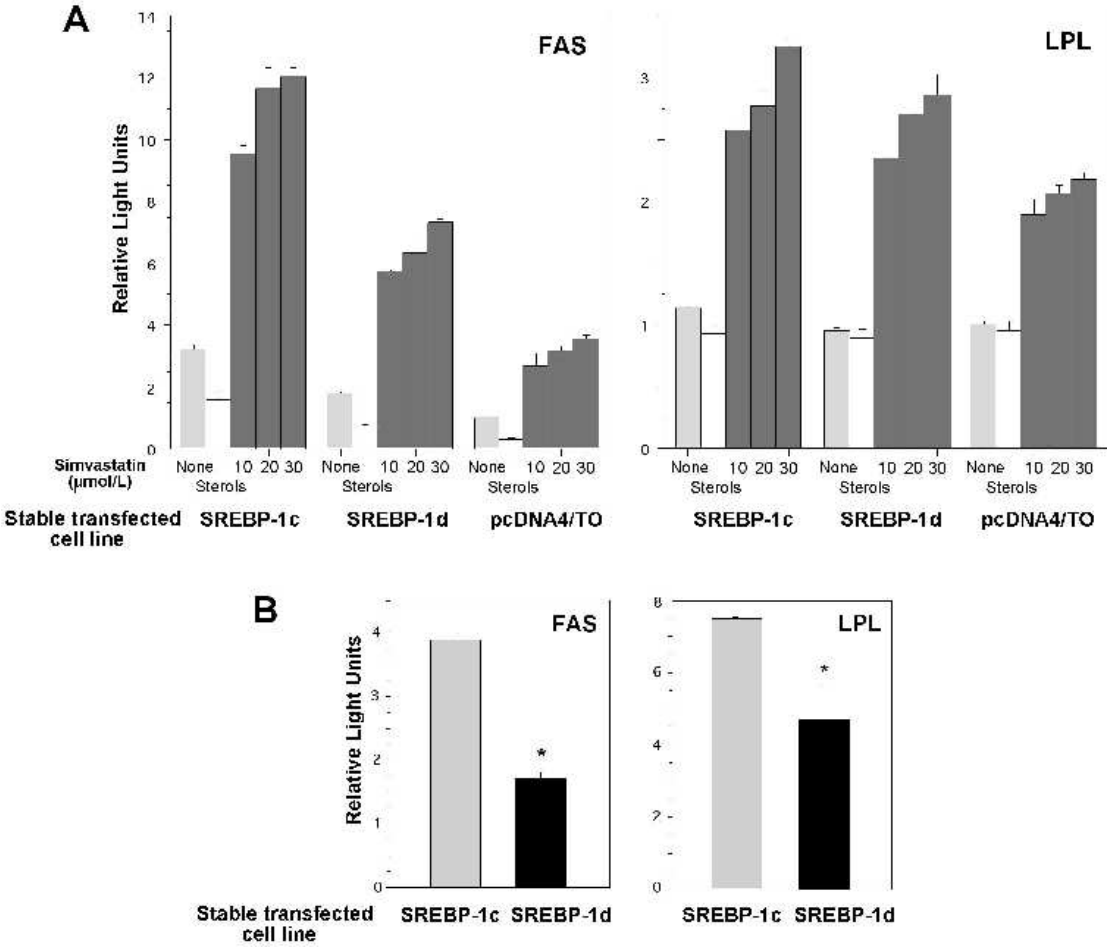

Figure 19. Characterization of the SREBP-1d stable transformant by transient transfection of luciferase reporter constructs. The luciferase reporter constructs containing the promoter region of fatty acid synthase (pGL2-*FAS*) and lipoprotein lipase (pGL2-*LPL*) were co-transfected with the pRL-CMV vector containing *Renilla* luciferase into T-REx cell lines, stably expressing either wild-type SREBP-1c or the splice variant SREBP-1d. The cell line stably transfected with the pcDNA4/TO vector

was used as control for tetracycline induction of endogenous SREBP-1. The sells were treated with 25-hydroxycholesterol (1 µg/ml) and cholesterol (10 µg/ml) (Sigma) as a control of the inhibition of the SREBP-regulated reporter genes; and with the dihydroxy-open form of simvastatin at final concentrations of $1x10^4$, $2x10^4$ and $3x10^4$ nmol/l as a control of the activation of the SREBP-regulated reporter genes. For all transfections, activation of luciferase was corrected for transfection efficiency with *Renilla* luciferase activity levels, and all data are represented accordingly as relative light units. **A**. Luciferase activity is expressed as the -fold increase relative to untreated (without simvastatin) control T-REx cells stably transfected with the pcDNA4/TO vector. **B**. Comparison of maximum activation of *FAS* and *LPL* reporter genes by simvastatin (30 µM) in T-REx cells stably expressing either wild-type SREBP-1c (grey) or SREBP-1d (black). Luciferase activity of control T-REx cell line was subtracted to luciferase activity of cell lines stably transfected with wild-type SREBP-1c and SREBP-1d. The *error bars* indicate S.D. from the mean. *, $P = 0.002$ (comparison between wild-type SREBP-1c and SREBP-1d for *FAS* gene); $P = 0.05$ (comparison between wild-type SREBP-1c and SREBP-1d for *LPL*).

It is important to notice, that expression of SREBP-1d even at low levels was sufficient to cause cell toxicity as observed by morphological changes of the cells. After induction of the stably transfected SREBP-1d splice variant with tetracycline for 16 hours, the cell toxicity was detected microscopically based on morphological alterations typical for adherent cells undergoing apoptosis, including becoming rounded, condensed, and detached from the dish. On the other hand, the cell toxicity was not detected in the cell line that was stably transfected with wild-type SREBP-1c after induction of expression of this isoform with tetracycline. This finding prompted us to assume that cell toxicity after induction of the splice variant SREBP-1d versus wild-type is dependent on differences exclusively in the last exons of the 3' terminus.

III.2.6. Differences of the mRNA stability of wild-type SREBP-1c and the splice variant SREBP-1d

For analysis of the SREBP-1d mRNA stability, T-REx-293 cells, stably transfected with wild-type SREBP-1c and the splice variant SREBP-1d, were cultured in the presence of tetracycline (1 µg/ml) for induction of these

isoforms. Transcription was then inhibited by the addition of actinomycin D. Actinomycin D was added to the growth medium (5 µg/ml), and the cells were incubated for the indicated time (Figure 20). Total cellular RNA from stably transfected cells was isolated. cDNA was used as a template for the quantitative polymerase chain reaction analysis based on the 5'-nuclease assay with the ABI PRISM 7000 Sequence Detection System (Applied Biosystems). Figure 20 demonstrates that mRNA of the splice variant SREBP-1d is more stable than wild-type SREBP-1c mRNA (P = 0.001). These data suggest that the exons differences in the coding 3'-end of the SREBP-1 gene affects the secondary structure of the SREBP-1 mRNA and influence its degradation rate.

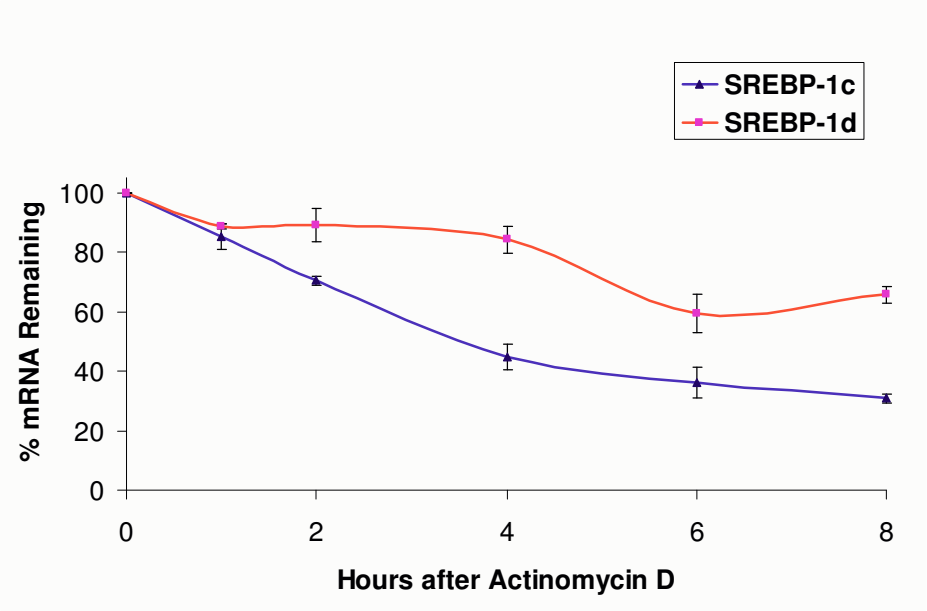

Figure 20. The mRNA stability of wild-type SREBP-1c and alternative splice variant SREBP-1d. T-REx-293 cells stably transfected with indicated expression constructs were treated for 16 hours with tetracycline (1 µg/ml). Than actinomycin D was added (5 µg/ml) and total cellular RNA was harvested after an additional 1, 2, 4, 6 or 8 hours. The SREBP-1 mRNA levels were evaluated with a real time quantitative polymerase chain reaction method as described in Materials and Methods. The *error bars* indicate S.D. from the mean. The *graph* depicts the % mRNA remaining after 8 h of actinomycin D.

IV. Discussion

IV.1. The role of SREBP-1c in drug induced hyperlipidemia

IV.1.1. The effects of HIV-protease inhibitor indinavir on SREBP-1c

In the present study, the influence of indinavir on the activation of effector genes involved in the triglyceride, cholesterol, and insulin metabolism was investigated.

The major findings were that indinavir (i) decreased the activities of the SREBP-1c/ADD-1-dependent *LPL* and *FAS* reporter genes in a dose-dependent manner and (ii) did not decrease the activity of the SREBP-1c/ADD-1-independent *LDLR* reporter gene.

Inhibition of the *LPL* and *FAS* reporter gene activities was detectable starting from an indinavir concentration of 10^{-1} nmol/l (*LPL*) or 10^{-2} nmol/l (*FAS*). For comparison: concentrations of 5×10^{1} to 10^{2} nmol/l indinavir inhibit viral spread by 95% in cell culture (Vacca *et al.*, 1994), concentrations of 10^{2}-10^{3} nmol/l indinavir correspond to physiological mean plasma concentrations in patients on treatment, and concentrations of 10^{3}-10^{5} nmol/l correspond to mean plasma concentrations usually not achieved in the steady state (Indinavir Sulfat. Merck, Sharp & Dohme; 1998). Since we observed clear decreases (12.4% for *LPL* and 30.3% for *FAS*) at an indinavir concentration of 10^{3} nmol/l, we expect that administration of indinavir in recommended doses (2.4 g daily) will result in changes in the expressions of the *LPL* and *FAS* genes *in vivo* as well. In contrast, a significant inhibition of the *LDLR* reporter gene activity was not detectable, even at an indinavir concentration of 2×10^{4} nmol/l. This result was not unexpected. We previously hypothesized that the pathophysiological mechanism to induce the hypertriglyceridemia, hypercholesterolemia, hyperinsulinemia, and lipodystrophy syndrome observed in HIV-infected patients is mediated by the SREBP-1c/ADD-1 pathway rather than by the SREBP-2 pathway (Miserez *et al.*, 2001). Since the gene encoding the *LDLR* is activated by SREBP-2 but much less by

SREBP-1c/ADD-1 (Shimano *et al*., 1997a), the lack of a significant inhibition of the *LDLR* reporter gene activity by indinavir is in line with our hypothesis.
Using other experimental approaches, PI have been shown to inhibit SREBP-1c/ADD-1 (Caron *et al*., 2001), insulin-stimulated glucose uptake (Murata *et al*., 2000), insulin signalling (Caron *et al*., 2001; Schütt *et al*., 2000) and adipocyte determination and differentiation (Caron *et al*., 2001; Zhang *et al*., 1999; Wentworth *et al*., 2000), and to induce adipocyte apoptosis (Domingo *et al*., 1999) and modulate proteosome activity (Schmidtke *et al*., 1999). In particular, the results of Caron *et al.* demonstrated a clear indinavir-induced impairment of SREBP-1 (Caron *et al*., 2001). Caron *et al.* focused on the indinavir-induced inhibition of insulin effects and adipocyte determination and differentiation, which are both mediated by SREBP-1c/ADD-1. Our results demonstrate the indinavir-induced inhibition of the lipoprotein and fatty acid metabolism, both also mediated by SREBP-1c/ADD-1, and are, therefore, in agreement with the observations of these authors. Thus, the indinavir-induced inhibition of effector genes regulated by SREBP-1c/ADD-1 can lead to hypertriglyceridemia, hypercholesterolemia, hyperinsulinemia and lipodystrophy: the syndrome observed in HIV-infected patients on ART.

SREBP-1c/ADD-1 plays a central role in the regulation of triglycerides, cholesterol, insulin, and adipose tissue formation. Administration of PIs, associated with hypertriglyceridemia, hypercholesterolemia, hyperinsulinemia and peripheral lipodystrophy, affects SREBP-1c/ADD-1 and, therefore SREBP-1c/ADD-1-dependent effector genes. SREBP-1c/ADD-1 specifically activates the genes encoding the LPL and FAS (Schoonjans *et al*., 2000; Bennett *et al*., 1995; Xiong *et al*., 2000; Magaña *et al*., 2000) as well as the adipocyte determination and differentiation (Tontonoz *et al*., 1993).
SREBP-1c/ADD-1 controls the peripheral clearance of triglyceride- and cholesterol-rich lipoproteins via regulation of *LPL* (Shimomura *et al*., 1998; Kim and Spiegelman, 1996; Shimano *et al*., 1996). Inhibition of *LPL* results in hypertriglyceridemia and hypercholesterolemia (Figure 21), as observed in inherited LPL deficiency syndromes (Figure 21) (Yang *et al*., 1995; Reynisdottir *et al*., 1995; Ishimura-Oka *et al*., 1992). SREBP-1c/ADD-1 controls fatty acid synthesis via regulation of *FAS* (Bennett *et al*., 1995;

Magaña *et al*., 2000) and the determination and differentiation of adipocytes (Tontonoz *et al*., 1993; Kim and Spiegelman, 1996). SREBP-1c/ADD-1 controls the insulin effect via regulation of a series of genes such as those encoding LPL (Schoonjans *et al*., 2000), FAS (Bennett *et al*., 1995; Magaña *et al*., 2000), and glucokinase (Foretz *et al*., 1999) (Figure 21). Inhibition of these genes results in a decreased insulin effect and, thus, insulin resistance (Foretz *et al*., 1999; Sul *et al*., 2000). Plasma insulin then increases in compensation (Flier and Hollenberg, 1999). Consequently, inhibition of *LPL*, *FAS* and the gene encoding the glucokinase results in hyperinsulinemia. The proof of this concept is inherited defects affecting, for example, the genes encoding LPL (Yang *et al*., 1995; Reynisdottir *et al*., 1995; Ishimura-Oka *et al*., 1992) and glucokinase (Froguel *et al*., 1993) (Figure 21). In line with these observations are our previous findings of a significant and parallel increase in plasma cholesterol and plasma insulin levels in HIV-1-infected subjects treated with PIs (Miserez *et al*., 2001).

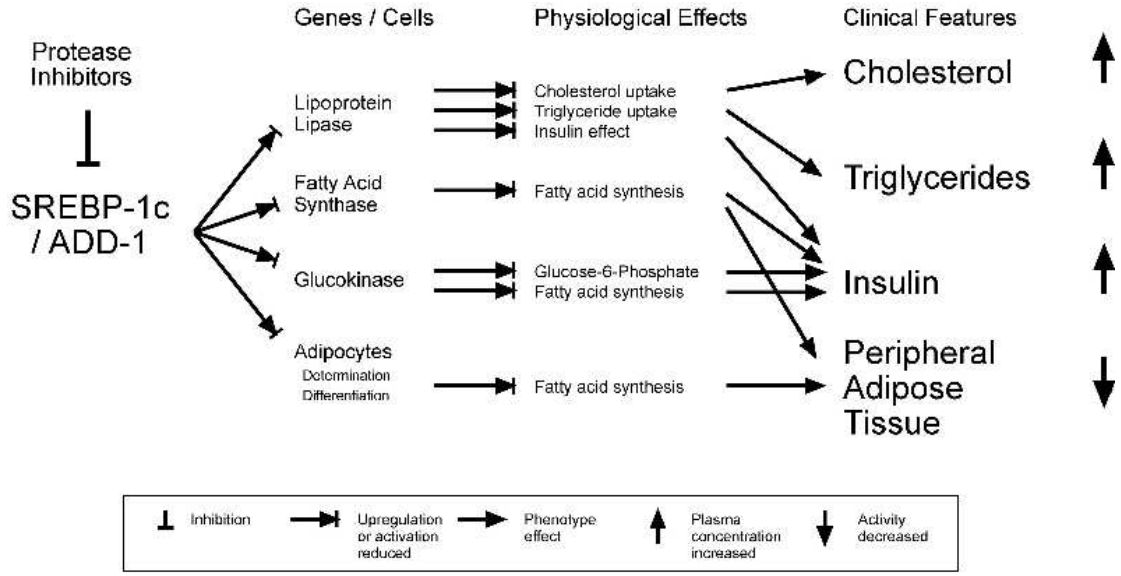

Figure 21. Protease inhibitor-induced changes in the sterol-regulatory element-binding protein-1c/adipocyte determination and differentiation factor-1c (SREBP-1c/ADD-1)-regulated pathway. SREBP-1c/ADD-1 controls the genes encoding the lipoprotein lipase (LPS), fatty acid synthase (FAS) and the glucokinase as well as the adipocyte determination and differentiation. In subjects treated with antiretroviral therapy (ART), inhibition of the SREBP-1c/ADD-1-mediated *LPL* activity is expected

to result in a combined hyperlipoproteinemia (increases in triglycerides and cholesterol) similar to that observed in LPL deficiency syndromes (Yang *et al*., 1995; Reynisdottir *et al*., 1995; Ishimura-Oka *et al*., 1992). Inhibition of SREBP-1c/ADD-1-mediated *FAS* activity is expected to result in a decrease in fatty acids and in an increase in insulin (Zhang *et al*., 1999; Wentworth *et al*., 2000). SREBP-1c/ADD-1 is regulated by insulin (Flier *et al*., 1999; Streicher *et al*., 1996; Shimomura *et al*., 1999) and has an insulin-mimicking effect (Foretz *et al*., 1999). Inhibition of SREBP-1c/ADD-1, therefore, results in decreased activity of various insulin-dependent genes (including the gene for glucose 6-phosphate; (Foretz *et al*., 1999; Flier and Hollenberg, 1999) and, hence, in reactive hyperinsulinemia. In ART-treated subjects, inhibition of SREBP-1c/ADD-1-mediated adipocyte determination and differentiation alters peripheral adipose tissue directly and may, therefore, contribute to ART-related peripheral lipodystrophy (Caron *et al*., 2001; Zhang *et al*., 1999; Wentworth *et al*., 2000).

Inhibition of fatty acid synthesis and adipocyte determination and differentiation results in decreased fatty acid synthesis and adipose tissue formation (Caron *et al*., 2001; Zhang *et al*., 1999; Wentworth *et al*., 2000) and might therefore explain particular aspects of peripheral lipodystrophy in humans (Figure 21). The mechanisms causing lipoatrophy however might rather be caused by additional pathways (TNF-α, PPAR-γ) (Bastard *et al*., 2002; Kannisto *et al*., 2003).

Proteolytic cleavage of SREBP-2 activates the gene encoding the LDLR strongly whereas proteolytic cleavage of SREBP-1c/ADD-1 activates *LDLR* much less (Shimano *et al*., 1997a). Similarly, simvastatin activates the SREBP-2-dependent *LDLR* gene strongly (approximately 500%, Fig. 8C) but activates the SREBP-1c/ADD-1-dependent *LPL* and *FAS* genes much less (approximately 100%, Fig. 8 A, B). Inhibition of the SREBP-2-dependent *LDLR* gene (as in familial hypercholesterolemia caused by *LDLR* gene defects) results in hypercholesterolemia but in most cases (90%) not in a hypertriglyceridemia (Brown and Goldstein, 1997). In ART-associated hyperlipidemia, however, hypertriglyceridemia is the predominant phenotype. In line with this latter observation, in our cell culture experiments, indinavir inhibited *LDLR* much less, indicating that the SREBP-1c/ADD-1-mediated

mechanisms play the predominant role in the development of ART-associated adverse effects.

We previously cloned and characterized the promoters of the differentially spliced genes of *SREBP-1*, *SREBP-1a* and *SREBP-1c/ADD-1* and characterized the *SREBP-2* gene (Miserez *et al*., 1997). Very recently, we identified in the gene encoding SREBP-1c/ADD-1 a marker predictive of the hypertriglyceridemia, hypercholesterolemia, hyperinsulinemia and lipodystrophy syndrome (Miserez *et al*., 2001). Although, in our experience, other cell lines such as HepG2 cells are more difficult to transfect than HEK293 cells, analogous experiments carried out using HepG2 cells instead of HEK293 cells resulted in similar inhibitory effects on *FAS* gene activation. Hence, there is strong evidence from others (Dowell *et al*., 2000; Caron *et al*., 2001) as well as from our own previous (Miserez *et al*., 2001) and present results that inhibition of SREBP-1c/ADD-1 is involved in the development of the ART-related hyperlipidemia, hyperinsulinemia, and lipodystrophy syndrome. Nevertheless, it remains to be clarified how PIs mediate the inhibition of SREBP-1c/ADD-1. The HIV-1 protease (GenBank 230883) and the site-1 protease (GenBank 4506775), a sterol-regulated protease that cleaves the SREBP molecules (Sakai *et al*., 1998), share a sequence homology at their catalytic sites. Despite this sequence homology, the HIV-1 protease and the site-1 protease belong to different classes of proteases. Therefore, because of considerable differences in the three-dimensional structures of the HIV-1 protease and the site-1 protease and based on steric models, it is unlikely that PI influence the SREBP pathway by direct inhibition of the site-1 protease. Similarly, these considerations apply to the hypothesis that PIs interact with the adipocyte-enhancer binding protein (Gagnon *et al*., 1998). Furthermore, if the site-1 protease was inhibited, we would expect quantitatively similar effects regarding the impairment of the activation of SREBP-1c/ADD-1- and SREBP-2-dependent genes. However, this was clearly not the case in our experiments.

A possible explanation for the differential inhibition of SREBP-1c/ADD-1- and SREBP-2-dependent genes is based on recent experiments suggesting differences in the regulation of the amount of mature SREBP-1c/ADD-1 and

SREBP-2. While SREBP-1c/ADD-1 is controlled by two independent mechanisms, by cleavage activation and by the rate of mRNA degradation (Xu *et al*., 1999), there is no evidence that SREBP-2 is regulated by the latter (Brown and Goldstein, 1997).

In summary, our demonstration of the inhibition of SREBP-1c/ADD-1-dependent genes explains the major metabolic effects described in association with ART.

IV.1.2. The role of single-nucleotide polymorphism in SREBP-1c gene in protease inhibitor-induced hyperlipoproteinemia

Inhibition of SREBP-1c-dependent genes by protease inhibitors such as indinavir explains the major metabolic effects associated with ART and demonstrates the crucial role of SREBP-1c in these metabolic pathways. A variant in the gene encoding SREBP-1c, the SREBP-1c-3'322C/G variant, was discovered as a pharmacogenetic marker that is predictive of HIV-PIs induced metabolic disturbances. Increases in cholesterol, triglycerides and insulin were less frequently associated with homozygous SREBP-1c-3'322G (genotype 22) than with heterozygous/homozygous SREBP-1c-3'322C (genotypes 11/12) (Miserez *et al*., 2001).

To investigate the molecular basis of these SNP-associated differences, we first excluded the possibility that SREBP-1c-3'322G causes alternative splicing. Silent polymorphisms/mutations (i.e. those that do not change the amino acid sequence, such as SREBP-1c-3'322C/G) may lead to alternatively spliced transcripts by disruption of exonic splicing enhancers (ESE) or activation of cryptic splicing sites and thus affect the correct splicing pattern (Maniatis and Tasik, 2002; Nissim-Rafinia and Kerem, 2002). We did not find an association between the new discovered alternative splicing of SREBP-1 at the 3' end of mRNA and the single-nucleotide polymorphism 3'322C/G in exon 18c.

Furthermore, synonymous mutations, such as SREBP-1c-3'322C/G, cannot be *a priori* considered as neutral or non-pathogenic, because they may directly affect mRNA structure and/or stability (Richard and Beckmann, 1995). The computer-based analyses was used to predict differences in the putative

mRNA secondary structure (SREBP-1c-3'322C versus SREBP-1c-3'322G). The analyses revealed conformational differences of SREBP-1c-3'322C/G isoforms at the mRNA level. This finding is particularly important because it has been demonstrated that the SREBP-1c pathway is regulated at the mRNA level additionally (Xu *et al*., 1999; Xu *et al*., 2001). As the expression of SREBP-1c gene can be regulated at the mRNA level, we supposed that another specific sequence variation (3'322C/G SNP) could affect the secondary structure of the SREBP-1c mRNA and influence its degradation rate additionally. A possible association between the mRNA stability of the SREBP-1c gene in the presence of a single-nucleotide polymorphism 3'322C/G was determined *in vitro*.

As it has been outlined before, the cells express two forms of SREBP-1, SREBP-1a and -1c. The SREBP-1 antibody recognizes both proteins. Consequently, the relative abundance of the two proteins cannot be differently quantified using Western blotting. Therefore, we compared the SREBP-1c mRNA stability of both homozygous genotypes using a real time quantitative polymerase chain reaction method using primers specific exclusively for the SREBP-1c isoform. For this study, the T-REx-293 System, a Tetracycline Regulated mammalian Expression System, was used. After having performed the stable transfection, stable cells lines were obtained which stably and inducibly expressed wild-type SREBP-1c (genotype 11) and SREBP-1c 3'322C/G (genotype 22). These stable cell lines provided an unique opportunity to study the influence of SREBP-1c SNP 3'322C/G on mRNA stability and to explain the changes in lipid metabolism in HIV patients after PIs treatment. Our results indicated that mRNA of the SREBP-1c-3'322G (genotype 22) was more stable than wild-type SREBP-1c-3'322C (genotype 11) mRNA. SREBP-1c-3'322C/G mRNA has a half-life of about 6 hours, whereas wild-type SREBP-1c has a half-life of about 3.5 hours. These data suggest that sequence differences in the coding 3'-end of the SREBP-1c gene (3'322C/G SNP) affect the secondary structure of the SREBP-1c mRNA and influence its degradation rate.

A striking observation was that the cell line which was stably transfected with SREBP-1c isoform contained the single-nucleotide polymorphism 3'322C/G activated SREBP-1c-dependent *FAS* and *LPL* reporter genes to a larger

extend than the cell line which was stably transfected with wild-type SREBP-1c isoform. Furthermore, these observations are in line with the SREBP-1c mRNA stability results obtained from real-time quantitative PCR experiments. The differences in genotype-specific SREBP-1c mRNA stability cause, therefore, the expression differences in the SREBP-1c-dependent genes.

Thus, the molecular mechanism of the 3'322C/G SNP-associated differences could be explained with different decay rates of both SREBP-1c mRNAs. A lower stability of SREBP-1c wild-type mRNA would result in an accelerated mRNA degradation and a weaker SREBP-1c activation. Hence, SREBP-1c would then be less active in presence of PIs in genotype CC/GG-carriers (genotypes 11/12) than in genotype GG-carriers (genotype 22). In the case of the wild-type SREBP-1c-3'322C isoform (genotype 11), fewer precursor molecules are cleaved and fewer mature transcription factor molecules enter the nucleus. This leads to a decrease in the activation of *FAS*, *LPL* and glucokinase genes. As a consequence, the cholesterol and triglyceride uptake from the plasma, fatty acid synthesis and insulin effects decrease. Subsequently, plasma total cholesterol, triglyceride, and insulin concentrations increase causing hypercholesterolemia, hypertriglyceridemia, and hyperinsulinemia in heterozygous/homozygous SREBP-1c-3'322C (genotypes 11/12) HIV-infected subjects after PIs treatment.

In summary, it has been demonstrated that sequence differences in the coding 3'-end of the gene (3'322C/G SNP) affect the secondary structure of the SREBP-1c mRNA, influence its degradation rate and, therefore, cause differences in the regulation of SREBP-1c expression.

IV.2. Alternative splicing of human SREBP-1

In the present study, we showed for the first time that there exist further functionally important splice variants of the human SREBP-1 gene. The new alternative splicing occurs at the 3' end of human SREBP-1 pre-mRNA and generates isoforms with large diversity of structure and function.

The resulting isoforms were different in the COOH-terminal domain of SREBP-1. It is important to notice that this COOH-terminal region performs

the essential regulatory function of the SREBPs (Brown and Goldstein, 1997). As it has been previously demonstrated, the newly synthesized SREBP is inserted into the membranes of the ER, where its COOH-terminal regulatory domain binds to the COOH-terminal domain of SCAP (SREBP cleavage activator protein). SCAP is both an escort protein for the SREBPs and a sensor of the intracellular sterol concentration. When cells are depleted of cholesterol, SCAP escorts the SREBP from the ER to the Golgi apparatus, where the SREBPs are cleaved. The N-terminal part of SREBP enters the nucleus and binds to sterol regulatory elements (SREs) in the promoters of genes encoding the enzymes of cholesterol and fatty acid biosynthesis (Brown and Goldstein, 1997; Horton *et al*., 2002). Truncation of SREBP at its COOH-terminus prevents the formation of complexes with SCAP and simultaneously reduces proteolytic cleavage (Sakai *et al*., 1997). Furthermore, the cytoplasmic COOH-terminal domain of the SREBP is required that the Site-1 enzyme can be active. When this domain in SREBP is shortened through truncation mutations, cleavage by the Site-1 protease is abolished (Ericsson *et al*., 1996). In this context it is important to notify that the new discovered alternative splicing changes the tertiary structure of the SREBP-1, especially the conformation of the COOH-terminus. It could play some functional role in such a way that the SCAP/SREBP complex formation, transport to the Golgi, cleavage (Site-1 protease) and, therefore, the release of active mature N-terminus isoform could be affected. As a result, the regulation of downstream genes of SREBP-1 (fatty acid synthase, lipoprotein lipase, glucokinase) and, subsequently, the metabolism of cholesterol, triglyceride, fatty acid, glucose, and insulin could be influenced.

In the splice variant SREBP-1c, exons 1, 18, and 19 are different from SREBP-1a (Hua *et al*., 1995), leading to dramatically different biological properties of SREBP-1c versus SREBP-1a (Shimano *et al*., 1997a). Cleavage of SREBP-1a protein, containing exons 18a and 19a in the COOH-terminal region, is completely suppressed by sterol overloading. However, in contrast, cleavage of SREBP-1c is not regulated by sterols (Hua *et al*., 1996). This finding was a surprise, and is very likely to have important physiologic implications. Sequences less important for regulation were traced back to two

exons of the COOH-terminal domain: 18a19a and 18c19c. An interesting finding is that the newly discovered alternative splicing event occurs exactly between these two exons. It may therefore influence the regulation of SREBP-1 (for example the sensibility to sterols) or the functional consequences of different splice isoforms.

In this context, it is important to mention that inducible expression of the splice variant SREBP-1d even at low levels was sufficient to cause cell toxicity as observed by morphological changes of the cells. On the other hand, the cell toxicity was not detected in the cell line that was stably transfected with wild-type SREBP-1c after induction of expression of this isoform with tetracycline. This finding prompted us to assume that cell toxicity after induction of the splice variant SREBP-1d versus wild-type is dependent on differences exclusively in the last exons of the 3' terminus.

Based on the observation that cell toxicity was detected after induction of the splice variant SREBP-1d only and not after induction of the wild-type SREBP-1c, we could explain a low level of expression of fatty acid synthase (*FAS*) and lipoprotein lipase (*LPL*) effector genes in the T-REx-293 cells stably transfected with the SREBP-1d isoform (Figure 19). Interestingly, after normalization to unstimulated cells, we observed an activation of *FAS* and *LPL* reporter genes by splice variant SREBP-1d larger than wild-type SREBP-1c. Additionally, the mRNA stability experiments in stably transfected T-REx-293 cells demonstrated that the mRNA of the splice variant SREBP-1d is more stable than the mRNA of wild-type SREBP-1c (Figure 20). This would cause an overexpression of the splice variant SREBP-1d and therefore an overexpression of lipogenic genes. It has been demonstrated in several animal models of diabetes that expression of SREBP-1c is increased in liver (Shimomura *et al*., 1999a; Kakuma *et al*., 2000). Overexpression of SREBP-1 has been suggested to be the cause of this kind of hepatic steatosis (Shimomura *et al*., 1999a; You *et al*., 2002). Furthermore, cell toxicity after expression of the nuclear active form of SREBP-1c (only N-terminus) was observed in insulinoma INS-1 cells (Wang *et al*., 2003). These results demonstrated that induction of the nuclear active form SREBP-1c leads also to INS-1 cells growth arrest and apoptosis (Wang *et al*., 2003).

Nonsense-mediated mRNA decay (NMD), is a mechanism by which cells can eliminate imperfect mRNAs, which would lead to the translation of potentially harmful or toxic proteins (Maquat, 1995). Nonsense and frameshift mutations introduce premature translation termination codons (PTCs) into the open reading frames (ORFs) of the affected mRNAs and are common causes of genetic disorders. The hallmark of NMD has been the reduction in abundance of the mRNA associated with a premature termination codon. Elimination of frameshifted mRNAs that result from cryptic splicing events is therefore likely to represent an important function of NMD in thalassemia and other genetic disorders (Danckwardt *et al*., 2002; Perrin-Vidoz *et al*., 2002). It should be mentioned that only one splice variant (SREBP-1e) could be appropriate to the mechanism of the nonsense-mediated mRNA decay. In mammals, a stop codon is recognized as a premature termination codon only when followed by an intron (Li and Wilkinson, 1998). Not all mRNAs that contain PTCs are target for destruction. As a general rule, only mammalian transcripts that contain a nonsense codon more than about 50 nucleotides upstream of the last exon-exon junction will be subjected to NMD (Zhang *et al*., 1998). Accordingly, the vast majority of mammalian genes contain the termination codon in the last exon or <50 nucleotides upstream of the last intron (Nagy and Maquat, 1998). This suggests that the nonsense-mediated mRNA decay requires an intron in the target mRNA. In the splice variant SREBP-1d a premature termination codon is located in exon 18c which followed an 3' untranslated region. This PTC can not be recognized and mRNA can not be subjected to NMD. In the case of splice variant SREBP-1e, the premature termination codon occurs in exon 18c which is followed by an intron and, subsequently, by exon 19c (Figure 14). This premature termination codon should be recognized as the trigger of NMD because it is located more than 50 nucleotides (67 nt) upstream of the intron or the final exon-exon junction. Interestingly, the mRNA of this splice variant was detected in the most human tissues (Figure 16) and lymphocytes from several patients. The presence of mRNA of this splice variant implies that the mutant message can escape nonsense-mediated mRNA decay (Perrin-Vidoz *et al*., 2002; Danckwardt *et al*., 2002) and that a truncated SREBP-1e protein may be produced in the cell. Moreover, this finding suggests the existence of additional, unidentified

determinants that modulate the NMD sensitivity of these transcripts (Danckwardt *et al*., 2002).

In the present study we investigated the expression patterns of the new SREBP-1 isoforms in different human normal and tumor tissues. Recently Shimomura I. *et al*. investigated the SREBP-1a and -1c expression in different human tissues and cultured cells (Shimomura *et al*., 1997a). They focussed on the expression of alternative versions of exon 1 of SREBP-1 only, which were designated 1a and 1c, respectively. They found that the 1a exon predominates in all cultured cell lines; in contrast, the 1c exon predominates in liver, adrenal gland, white adipose tissue, ovary, brain and muscle tissues. Because the expression pattern of SREBP-1 at the 5' end was known (Shimomura *et al*., 1997a), we concentrated on the 3' end of SREBP-1 mRNA. Surprisingly, SREBP-1c, -1d, -1e and -1f isoforms were expressed differently in various tissues. The alternatively spliced SREBP-1e transcript was expressed in all normal tissues and tumor-derived cells at different levels. The highest expression levels were detected in liver, placenta, pancreas, testis, thymus and prostate tissues, and in lung carcinoma (LX-1 and GI-117), colon adenocarcinoma (CX-1 and GI-112) and ovarian carcinoma (GI-102) in the tumor-derived cells. The SREBP-1d transcript was detected in most of the normal tissues and tumor-derived cells examined at different expression levels. The highest expression was detected in liver and testis, and lung carcinoma (LX-1 and GI-117) in the tumor-derived cells. On the other hand, the SREBP-1f splice variant was detected only in peripheral blood leukocyte with a low expression level. The analysis detected wild-type SREBP-1c transcript with low expression level in placenta, lung, spleen and thymus, and in all tumor-derived cells with exception of prostatic and pancreatic adenocarcinoma (PC3 and GI-103, respectively). SREBP-1e and SREBP-1d showed a much broader tissue distribution, whereas the expression of SREBP-1c and, especially, SREBP-1f were relatively tissue-specific. It is important to mention that the splice variant of human SREBP-1c (wild-type) which sequence is published in GenBank (AH004383), was isolated from cultured HeLa cells (Yokoyama *et al*., 1993) where this transcript is most abundant. Whereas in our study the new splice variants were isolated from

human normal tissues and tumor-derived cells where these transcripts, in particular SREBP-1d and -1e, were most abundant. This high abundance led us to the hypothesis that SREBP-1e and SREBP-1d play a more general role in regulating cellular lipid levels as compared to other isoforms.

Based on the finding of three new isoforms of the SREBP-1 gene, we were curious to investigate whether certain drugs, that have an influence on SREBP-1c, could induce or suppress their expression. We confirmed that upon HepG2 cells stimulation by simvastatin, indinavir, insulin and LPDS (conditions with low lipoprotein concentration) the mRNA pattern of SREBP-1 splice variants was changed. Conditions with low lipids concentration (LPDS medium) markedly increased the level of SREBP-1e mRNA. After treatment with indinavir the expression of SREBP-1e mRNA was also induced. In contrast, the SREBP-1d mRNA was moderately decreased. The splice variants SREBP-1d and SREBP-1e were induced by treating cells with insulin. Interestingly, when HepG2 cells were treated with the lipid lowering drug simvastatin (30 µM), the mRNA level of all SREBP-1 variants was markedly decreased (Figure 18). The mRNA expression levels of SREBP-1c and SREBP-1f were not much affected by changing of conditions or adding drugs. The results collectively demonstrated that the induction pattern of mRNA of SREBP-1 splice variants were clearly different from each other after addition of certain drugs (simvastatin, indinavir, insulin), that affect the SREBP-1c expression, or changing the lipid concentration. Therefore, the choices of the splice sites occur in highly inducer-specific manners. However, because of the general lack of understanding how cells choose and change particular splice sites (Black, 2000), regulatory mechanisms of alternative splicing of the SREBP-1 gene are still unclear.

Our results pointed to the direction that alternative splicing may be a mechanism in regulating SREBPs functions in different tissues and cells types. The marked divergence of SREBP-1 isoforms elicited by alternative splicing may reflect their physiological importance and the needs to respond to a variety of signaling pathways operating under distinct regulatory mechanisms.

V.Outlook

The aim of any drug treatment is to achieve the desired effect while having few side effects. Genetic differences between individuals can cause differences in the efficacy of a particular drug and can contribute to unexpected side effects. The sterol-regulatory element-binding protein-1c became an interesting candidate gene, based on the pattern of physiological processes it regulated and being possibly involved in the pathogenesis of the metabolic syndrome. The genetic marker SREBP-1c-3'322C/G is associated with hypertriglyceridemia, hypercholesterolemia, hyperinsulinemia, and lipodystrophy syndrome, which can occur during therapy with antiretroviral drugs as part of an antiretroviral treatment (ART), in particular when protease inhibitors are used. The pharmacogenetic basis of this finding can be explained by the differences in messenger RNA stability of both genotypes (SREBP-1c-3'322C versus SREBP-1c-3'322G). Hence, this novel genetic marker can be routinely tested before the specific drug is taken and can contribute to the optimization of the efficacy and reduction of side effects.

In addition, a variety of new splice variants of the SREBP-1 gene has been described as well as a number of drugs (for example: indinavir, simvastatin) on their expression level has been examined. The present study supports the hypothesis that the response to a certain drug can alter the splice variant pattern of SREBP-1. Furthermore, RNA derived from the lymphocytes of the various individuals investigated demonstrated a considerable variety of splice variants in different quantities. Based on the fact that different drugs have various effects and side effects in different patients, it is important to create further tools which: (i) allow the prediction of possible adverse effects of drugs in patients by altering their SREBP-1 pattern, and (ii) if there is a side effect of a given drug, some patients may be protected from particular adverse effects. Further studies could investigate the additional functions of SREBP-1 splice variants. Further insights how the SREBP-1 isoforms interact with the signal transduction pathways, and how these signals regulate the splicing pattern and mRNA stability of these variants are the goal of further research projects.

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VII. Appendix

VII.1. Curriculum vitae

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Education

2000-2004	PhD thesis Institute of Biochemistry and Genetics Cardiovascular Genetics
1997-1999	M.Sc. degree, Vilnius University, Department of Biochemistry, Lithuania
1993-1997	B.Sc. degree, Vilnius University, Department of Biochemistry, Lithuania

Work Experience

1997-2000	Research assistant, Vilnius University, Department of Biochemistry, Lithuania. Field: Molecular Biology and Microbiology

VII.2. Publications

Saudinyte V., Suziedeliene E. and Juodka B. Transcriptional analysis of acid-inducible *E. coli asr* gene: the roles of pH, growth phase and RpoS sigma factor. *Biology* (Lithuania) 1998; **1**(1): 45-47.

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During PhD thesis I attended lectures of following lecturers:

M. Affolter, K. Balmer-Hofer, E. Battegay, C. Beglinger, M.M. Burger, R. Clerc, G. Christofori, J. Drewe, A.N. Eberle, M. Hall, J. Heim, M. Heim, T. Hohn, G. Hollaender, N. Hynes, W. Keller, S. Kraehenbuehl, P. Matthias, U.A. Meyer, A.R. Miserez, C. Moroni, H. Mueller, U. Otten, P. Philippsen, T. Resink, A.G. Rolink, R. C. Skoda, M. Spiess.