INSIGHTS INTO EXPRESSION, CELLULAR LOCALIZATION, AND REGULATION OF SUPERNATANT PROTEIN FACTOR, A PUTATIVE REGULATOR OF CHOLESTEROL BIOSYNTHESIS

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ABSTRACT OF DISSERTATION

Elzbieta Ilona Stolarczyk

The Graduate School
University of Kentucky
2009
INSIGHTS INTO EXPRESSION, CELLULAR LOCALIZATION, AND REGULATION OF SUPERNATANT PROTEIN FACTOR, A PUTATIVE REGULATOR OF CHOLESTEROL BIOSYNTHESIS

ABSTRACT OF DISSERTATION

A dissertation submitted in partial fulfillment of the requirements of the degree of Doctor of Philosophy in The Graduate School at the University of Kentucky

By
Elzbieta Ilona Stolarczyk
Lexington, Kentucky

Director: Dr. Todd D. Porter, Associate Professor, Pharmaceutical Sciences
2009
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ABSTRACT OF DISSERTATION

INSIGHTS INTO EXPRESSION, CELLULAR LOCALIZATION, AND REGULATION OF SUPERNATANT PROTEIN FACTOR, A PUTATIVE REGULATOR OF CHOLESTEROL BIOSYNTHESIS

SPF (Supernatant Protein Factor) is a cytosolic protein that stimulates at least two enzymes in the cholesterol biosynthetic pathway: squalene monooxygenase and HMG-CoA reductase. The mechanism of action has not been established but may be related to lipid transfer between intracellular membranes.

There are three human genes for SPF: SEC14L2 (SPF1), SEC14L3 (SPF2) and SEC14L4 (SPF3). The present study differentiates these closely related genes by evaluating their tissue-specific and relative expression levels. SPF1 mRNA was found to be most abundant in liver, mammary gland and stomach. SPF2 showed negligible expression in all tissues tested; SPF3 expression pattern was similar to that of SPF1, but at 10-50-fold lower levels than SPF1.

A cDNA to SPF3 was cloned and, upon transfection into rat hepatoma cells, was shown to increase cholesterol synthesis by approximately 50%, similar to that obtained with SPF1. However, in contrast to SPF1, SPF3 did not stimulate squalene monooxygenase activity in microsomal preparations, suggesting that it acts primarily through activation of HMG-CoA reductase.

SPF possesses a lipid binding domain (Sec14) and a Golgi dynamics domain (GOLD). SPF resides in the cytosol and requires phosphorylation and the presence of Golgi in order to stimulate cholesterol synthesis. To determine if SPF associates with specific subcellular structures, cellular immunofluorescence studies were carried out. A phosphorylation-defective mutant, a protein lacking the GOLD domain, and the effect of protein kinase A-mediated phosphorylation of endogenous SPF were examined. No change in the subcellular location of SPF could be detected with either the phosphorylation mutant or the native SPF after protein kinase A activation. However, removal of the GOLD domain resulted in a protein that co-localized with large vesicular structures around nucleus.
Studies with rat hepatoma cells showed that the expression of the two rat SPF genes is upregulated in response to serum deprivation, and is potentiated by removal of glucose. Lipid/cholesterol availability was demonstrated to be at least one of the serum components that affected SPF transcript levels. The oxysterol receptor LXR was shown not to be involved in SPF gene regulation, implicating SREBP and/or PPARα as the principal regulators of SPF gene transcription.

KEYWORDS: Supernatant Protein Factor, Cholesterol, Lipid Binding Domain, Golgi Dynamics Domain, Immunofluorescence
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For My Friend Luis Hedian
ACKNOWLEDGMENTS

The following dissertation, while an individual work, benefited from the insights and direction of several people.

First, I would like to thank my mentor, Dr. Todd Porter, for his guidance and support throughout my doctoral work. He patiently provided the vision, encouragement and advice necessary for me to proceed through the doctoral program and complete my dissertation. Dr. Porter continually stimulated me to develop independent thinking and research skills, and greatly assisted me with writing. I would especially like to thank him for sharing his optimism and positive outlook, which I often lacked, and without which completion of my dissertation would be impossible. I am confident that I will continue to benefit from his influence in my career and personal life.

Next, I wish to thank the members of my dissertation committee, Dr. Daniel Tai, Dr. Peter Wedlund and Dr. Mariana Nikolova-Karakashian for their support and helpful suggestions throughout my studies. I would also like to thank Dr. Douglas Andres for agreeing to be my outside examiner for my dissertation defense.

Special thanks to Dr. Gregory Graf who has provided helpful insights as well as technical assistance critical for completing my research project.

In addition, I would like to thank Dr. Dejan Nikolic and my current and former colleagues in Dr. Porter’s and Graf’s laboratories: Dr. Lee Elmore, Dr. Li Li, Subhashis Banerjee, Nadezhda Sabeva and Eric Rouse for their technical assistance, support and friendship.

Finally, I would like to thank my family and friends for their faith in me and continuous support during my doctoral studies.
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Chapter 1: INTRODUCTION

Why is cholesterol important?

It has been proposed that the availability of molecular oxygen gave rise to eukaryotic life and significantly impacted the evolution of species \(^1\). Konrad Bloch, who was awarded a Nobel prize for delineating the enzymatic steps in the cholesterol biosynthetic pathway, showed that it was very unlikely for squalene to become cyclized to lanosterol without molecular oxygen and even less likely for subsequent conversion of lanosterol to cholesterol \(^1\). Therefore it has been postulated that availability of molecular oxygen made the synthesis of cholesterol possible. Bloch, impressed with the particular sequence of systematic enzymatically controlled reactions following cyclization of squalene to lanosterol, proposed that evolutionary pressure demanded the formation of cholesterol to satisfy the physical requirements of plasma membranes in eukaryotic cells. In fact, the influence of various sterols on membrane “microviscosity” was examined in vitro and a systematic increase in membrane microviscosity was found progressing from lanosterol to cholesterol \(^1\). It was also postulated that certain membrane-related events unique to eukaryotes, such as endo-and exo-cytosis, became possible due to the appearance of cholesterol. Taken together, cholesterol is viewed as a crucial molecule in the evolution of membranes and, moreover, eukaryotic life \(^1;2\).

Cholesterol markedly influences plasma membrane thermomechanical properties \(^2\). As an amphipathic molecule, cholesterol is able to intercalate into the lipid bilayer with its small hydrophilic head group and increase lipid order in fluid membranes to maintain fluidity and alter the phase transition. Cholesterol also increases bilayer thickness and
decreases membrane permeability. Cholesterol also plays an important role in lateral organization of the membrane by formation of lipid rafts, which are areas of specific lipid-protein composition involved in signal transduction. The cholesterol molecule itself is involved in cellular signaling, in that certain proteins respond to cholesterol levels through their sterol sensing domains, to influence cholesterol synthesis and metabolism. In addition, early in development certain signaling molecules from the Hedgehog family of morphogens are covalently modified by cholesterol.

A variety of physiologically important molecules are derived from cholesterol: Vitamin D, which maintains calcium homeostasis; bile acids, which are necessary for solubilization and absorption of dietary fats; sex steroids responsible for sexual differentiation and maintenance of reproductive functions; and the hormones of the adrenal cortex: mineralocorticoids, involved in salt and water homeostasis, and glucocorticoids, regulators of growth, metabolism, and the response to stress and inflammation.

Apart from cholesterol, some intermediates of the pathway serve as precursors for the synthesis of various non-steroidal isoprenoids. For example, farnesyl or geranylgeranyl groups anchor certain proteins to the membrane (Ras, Rho, Rab etc.); dolichol takes part in glycoprotein synthesis; and ubiquinone and heme A are constituents of the respiratory chain complexes.

In higher eukaryotes cholesterol is either synthesized in cells, with liver and intestine being major sites of production, or is absorbed in the gut from food. Blood transports cholesterol in the form of lipoprotein particles. Cholesterol absorbed from food is transported from the small intestine to peripheral tissues and ultimately the liver as chylomicron particles. Exogenous and newly synthesized cholesterol constitutes the liver’s pool of cholesterol. Hepatocytes utilize cholesterol by incorporating it into the plasma membrane and for bile acid synthesis. Free cholesterol is esterified, packaged into VLDL particles and secreted into the bloodstream. In blood, VLDL particles are
reduced to more dense LDL particles and in this form cholesterol is distributed throughout the body. In peripheral tissues cholesterol is taken up by cells via LDL or scavenger receptors. Cells incorporate it into the plasma membrane and may use it for the synthesis of steroid hormones. Excess cholesterol is incorporated into HDL lipoproteins and for transport back to the liver. The liver is able to take up all lipoprotein particles: chylomicrons, LDL and HDL. Bile acids produced in the liver are secreted into the bile and stored in the gallbladder to be released into intestine to solubilize dietary lipids. Bile acids are then either reabsorbed and returned to liver or excreted. Excretion of bile acids is the only route of cholesterol removal from the organism.

Maintenance of a balanced lipid profile is crucial for health. Elevated levels of LDL and low level of HDL lipoproteins are associated with atherosclerosis and coronary heart disease, a major cause of death in western societies.

**Cholesterol biosynthetic pathway**

The cholesterol biosynthetic pathway is of major importance for animal and human physiology. It gives rise to cholesterol, steroidal derivatives and non-steroidal isoprenoids.

Cholesterol synthesis occurs in eukaryotic cells from the two carbon molecule acetate, esterified to coenzyme A, in a series of condensation reactions. In cytosol three molecules of acetyl-CoA are condensed to form 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) which is then reduced to form mevalonate. This reduction is catalyzed by HMG-CoA reductase and it is the principal regulatory step in the pathway. Subsequently, mevalonate is phosphorylated to form several active intermediates and then decarboxylated to form the 5-carbon isoprenoid isopentenyl pyrophosphate (IPP). IPP is
in equilibrium with its isomer dimethylallyl pyrophosphate (DMPP) and these two molecules condense to form geranyl pyrophosphate (GPP). GPP condenses with another molecule of IPP to yield farnesyl pyrophosphate (FPP), a 15-carbon molecule. Squalene synthase then catalyzes the condensation of two FPP molecules to yield squalene, a 30-carbon molecule that is subsequently oxidized to 2,3-oxidosqualene by squalene monooxygenase in presence of molecular oxygen and NADPH. This epoxidation of squalene is the first oxygen-requiring step in cholesterol synthesis, and is followed by a cascade of electron shifts resulting in cyclization, all catalyzed by oxidosqualene cyclase and yielding the characteristic steroid ring structure: lanosterol. Conversion of lanosterol to cholesterol involves 19 steps, including a series of reduction and demethylation reactions, all taking place in the membranes of endoplasmic reticulum.
Figure 1.1: Cholesterol biosynthetic pathway.
Stimulatory effect of supernatant fluid on cholesterol biosynthesis

The first report on the ability of “supernatant fluid” (i.e., the 100,000 x g supernatant fraction of a tissue homogenate, equivalent to the cell cytosol) to activate the cyclization of squalene to lanosterol appeared during an investigation on cholesterol biosynthetic pathway in the Konrad Bloch laboratory. This cyclization occurs in two steps; first squalene monooxygenase epoxidizes squalene to 2,3-oxidosqualene, followed by cyclization to lanosterol catalyzed by oxidosqualene cyclase. It was shown with microsomal assays that the liver 100,000 x g supernatant fraction stimulates membrane-bound squalene monooxygenase. Subsequently it was determined that this supernatant fraction provides two essential components, one heat-stable and one heat-labile. The heat-labile component proved to be a protein, which was purified and termed Supernatant Protein Factor (SPF). The heat-stable components of the supernate could be replaced by anionic phospholipids (phosphatidylinositol, phosphatidylserine, phosphatidylglycerol) and FAD. Solubilized squalene monooxygenase, while enzymatically active, did not respond to SPF and phospholipids, and therefore it was concluded that intact membrane is required to observe this stimulation. The apparent requirement for an intact membrane suggested a substrate- or product-carrier function for SPF, but binding to substrate (squalene) or product (2,3-oxidosqualene) could not be shown. Later it was found that SPF also stimulates the subsequent step, conversion of squalene 2,3-epoxide to lanosterol, mediated by oxidosqualene cyclase. In this light, SPF seemed to influence intramembrane events rather than act as a carrier protein. Presumably, together with anionic phospholipids, SPF affects some membrane properties or membrane-associated events which influence the activity of these enzymes. In 1980 Friedlander et al. demonstrated that SPF facilitates the intramembrane transfer of squalene in in vitro preparations. The mechanism by which this cytosolic protein directs movement of lipophilic substrate across or between
bilayers remained obscure, but it was proposed that it must involve direct interaction with the surface of another membrane or particle\textsuperscript{10}.

### Structural features of SPF

In 2000 a novel human ‘tocopherol-associated protein’ (TAP) was cloned, expressed, and characterized\textsuperscript{11}, soon after the cloning of human SPF was reported\textsuperscript{12, 13}. It wasn’t recognized until later that TAP and SPF are in fact the same entity\textsuperscript{13}. Supernatant Protein Factor/SEC14-like protein 2/Alpha-Tocopherol-Associated Protein (SPF/Sec14l2/TAP) is a 403-amino acid protein found in the cytosol of mammalian cells. The determination of the SPF primary sequence allowed for sequence comparison studies; a BLAST alignment with the database of known protein sequences revealed conserved domains within Sec14l2 protein (SPF) as depicted in the schematic figure below (Fig.1.2)

![Figure 1.2: Conserved domains of SPF protein.](image)

Soon after cloning and expression of SPF, the three-dimensional structure was published (Fig. 1.3.)\textsuperscript{14}. 

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\[\text{Figure 1.2: Conserved domains of SPF protein.}\]
Figure 1.3: Crystal structure of human supernatant protein factor as a ribbon diagram.

The lipid binding cavity is shown in grey, the CRAL/TRIO_N domain in navy, the Sec14 domain in turquoise-green, and the GOLD domain in yellow-orange (as on the scheme in Fig. 1.2). Taken from reference 14.

Further sequence alignments with the database found presumed homologues in several species and various proteins that share the same domains. There are three highly similar SPF proteins in the human genome: hSPF1 (TAP1, Sec14-like 2 protein), hSPF2 (TAP2, Sec14-like 3 protein), and hSPF3 (TAP3, Sec14-like 4 protein). Two proteins in the rat genome also show high similarity: rSPF (rSec14l2) and p45 (rSec14l3, rat SPF-like protein), a 45-kDa secretory protein from rat olfactory epithelium.

Sec14-like proteins were named after their main protein module: **Sec14 domain** (smart00516). Sec14 is a conserved domain known to bind small lipid molecules, including phosphatidylinositol, retinol, and α-tocopherol. This domain is found in a number of secretory proteins, such as *S. cerevisiae* phosphatidylinositol transfer protein...
(Sec14p), and multiple lipid-regulated proteins: Cellular Retinal Binding Protein (CRALBP), Tocopherol Transfer Protein (α-TTP), RhoGAPs, RhoGEFs, PTPMEG2, and others. The Sec14 domain of PTPMEG2 was found to contain motifs necessary for targeting to the secretory vesicles\(^\text{15}\), which might be an additional function besides lipid binding that this domain possesses. The all-α N-terminal portion of this domain was recently annotated in the NCBI database as a separate protein module: **CRAL/TRIO_N domain** (pfam03765); this domain is absent in some members of the Sec14 family, such as CGR-1. The carboxy-terminal part of Sec14-like proteins contains a β-strand-rich protein module, named the **GOLD domain** (for Golgi Dynamics) (pfam01105). This domain is proposed to mediate protein-protein interactions. The GOLD domain of the GCP60 protein was shown to interact with Golgi residual protein giantin\(^\text{16}\). It is the main domain of members of the emp24/gp25L/p24 family, which are proteins involved in Golgi processing and secretion. P24 proteins are critical components of coated vesicles, bringing cargo forward from the endoplasmic reticulum to the Golgi. The GOLD domain is always found combined with a lipid-binding domain (CRAL/TRIO, Sec14, or OxB) or with membrane-association domains (PH, FYVE)\(^\text{17-19}\). Interestingly, a recently characterized CGR-1 protein from *C. elegans* is the only other known protein that, like the SPF proteins, contains both Sec14 and GOLD domains. CGR-1 positively regulates Ras signaling during *C. elegans* vulvae development\(^\text{20}\).
The availability of the SPF nucleotide sequence facilitated the cloning of a cDNA to SPF and expression of the recombinant protein. Recombinant, bacterially expressed SPF was able to stimulate the transfer of squalene and activity of squalene monooxygenase in microsomal preparations. Hepatoma cells transfected with a plasmid carrying the SPF cDNA expressed the recombinant human protein and showed a 2-3 fold increase in cholesterol synthesis, extending earlier studies with purified proteins in reconstituted systems.
Three human SPF genes

As noted above, there are three highly similar genes encoding SPF-like proteins in the human genome: SPF1, SPF2 and SPF3. The degree of identity to SPF1 at the protein level is 77% for SPF2 and 70% for SPF3, as shown in Fig. 1.4. The degree of identity to SPF1 at the mRNA level is 66% for SPF2 and 59% for SPF3 (full alignment of these transcripts is shown in Fig. 2.1). The SPF protein that has been the most studied so far is SPF1 (NP_036561), also referred to as SEC14-like 2, tocopherol-associated protein (TAP), or simply SPF. Human SPF2 (NP_777635) is also called SEC14-like 3, and SPF3 (NP_777637.1) is SEC14-like 4.

Stimulation of HMG-CoA Reductase by SPF

Studies in the 1970’s and 1980’s indicated that SPF stimulated the conversion of squalene to lanosterol. This conversion can be broken down to epoxidation of squalene to 2,3-oxidosqualene catalyzed by squalene monooxygenase, and cyclization of oxidosqualene to lanosterol catalyzed by oxidosqualene cyclase. As mentioned earlier, SPF was shown to stimulate both enzymes. Later, studies with hepatoma cells transfected with SPF showed that the increase in cholesterol synthesis when measured with [14C]-mevalonate was less than that obtained with [14C]-acetate. This unexpected finding suggested that part of the SPF stimulation was at or upstream of HMG-CoA reductase, the enzyme that generates mevalonate. To explore this possibility, terbinafine, an inhibitor of squalene monooxygenase, was used to block the conversion of squalene to cholesterol in SPF-transfected cells; under these conditions the synthesis of squalene was increased when measured from [14C]-acetate and was unchanged when measured with [14C]-mevalonate. This finding further indicated a stimulatory effect of
SPF on mevalonate synthesis. There are only 3 steps in the pathway between acetate and mevalonate: the condensation of two acetate molecules catalyzed by β-ketothiolase, followed by synthesis of 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) by HMG-CoA synthase, and reduction of HMG-CoA mediated by HMG-CoA reductase. A stimulatory effect on HMG-CoA reductase was confirmed in microsomal assays by measurement of mevalonate synthesis from HMG-CoA in presence or absence of SPF protein 23. Although it was not investigated if β-ketothiolase or HMG-CoA synthase is also affected by SPF, it was suggested that HMG-CoA reductase is the main target, as this is the main regulatory enzyme of the pathway 23.

**Requirement for Golgi in SPF function**

Knowledge of SPF structural motifs has allowed for structure-function studies to be undertaken. Deletion of the GOLD domain resulted in a protein that was no longer able to stimulate cholesterol synthesis 22. The GOLD domain has been suggested to be involved in interaction with Golgi, and therefore it was argued that an interaction of SPF with Golgi was necessary for SPF stimulatory effect. This suggestion was supported by studies with brefeldin-A, which disrupts Golgi structures: The stimulatory effect of SPF on cholesterol synthesis was abolished when Golgi were disrupted with this agent 22. Moreover, washout of brefeldin-A, which allows for Golgi reassembly, resulted in a regain of SPF activity. This clearly demonstrated that SPF requires Golgi for its function, although the nature of the interaction between SPF and Golgi remains to be elucidated.
SPF activation by phosphorylation

It was observed in our laboratory that addition of ATP to microsomes increases the stimulatory effect of SPF on squalene monooxygenase. This finding was further explored by treatments with a variety of protein kinase inhibitors and protein kinases themselves. It was concluded that both protein kinase A and C are capable of SPF phosphorylation. Cell culture studies with dibutyryl-cAMP, a PKA stimulator, confirmed that SPF needs to be activated by PKA to achieve its full activity. Cell-permeable protein kinase A inhibitor 14-22 amide was further able to prevent this activation of SPF. Phosphorylation appeared to increase the SPF stimulation of both squalene monooxygenase and HMG–CoA reductase. Moreover, sequence analysis of SPF revealed the presence of a putative protein kinase A (PKA) phosphorylation site within the coding sequence. Substitution of the serine in position 289 with alanine resulted in a protein unable to stimulate cholesterol synthesis in cell culture when compared with wild-type protein.

Dose response curve for SPF

In vitro assays showed that the amount of SPF used in the assay is critical. SPF activity increases with an increase in the amount of protein present, reaching its maximum at 0.8 μg per incubation, and then quickly declining, resulting in total loss of stimulation. It was shown that the SPF activation curve is similar for both enzymes, squalene monooxygenase and HMG–CoA reductase.

These two observations of similar behavior of SPF led to the suggestion that the mechanism of stimulation is similar for both enzymes. Since SPF stimulates at least three enzymes, HMG-CoA reductase, squalene monooxygenase, and oxidosqualene...
cyclase, it can be argued that the mechanism by which SPF acts is through a general, membrane-dependent event.

SPF-null mice

Despite the many possible roles for SPF in cellular and organismal biology, SPF (-/-) null mice did not show an abnormal phenotype, and developed normally 24. Notably, plasma lipids levels were unchanged despite elevated levels of HMG-Co A synthase and squalene monoxygenase in liver. However, during fasting plasma cholesterol levels decreased significantly in SPF-null mice, but not in wild-type mice. Fasting decreased hepatic cholesterol synthesis in both wild-type and SPF-null animals, but with a greater reduction in SPF-null mice. The decrease in cholesterol synthesis during fasting was accompanied by a pronounced decrease in the expression of cholesterolgenic enzymes: HMG-CoA synthase, HMG-CoA reductase and squalene monoxygenase in both wild-type and SPF-null mice. Interestingly, in fasted wild-type mice, the expression of SPF mRNA and protein was elevated. Since PPARα is known to play a role in hepatic response to fasting 25, 26, PPARα-null mice were subjected to fasting and did not show an increase in SPF expression upon treatment. It was therefore concluded that the increase in SPF protein expression during fasting is dependent on PPARα.

The data from SPF-null mice strongly suggests that SPF is upregulated upon fasting to compensate, at least partially, for the decrease in cholesterol synthesis. The reason for this compensatory increase is not apparent. Treatment with fibrates, which are PPARα agonists, also resulted in upregulation of SPF protein expression. The same treatments in PPARα-null mice did not elevate SPF, indicating a PPARα dependency. Fibrates are used to lower plasma triglyceride levels; here it was shown that administration of fibrates to SPF-null mice resulted in significant reduction in plasma cholesterol levels as
well, suggesting that coadministration of a fibrate and an SPF antagonist, if one can be discovered, could be beneficial for improving the plasma lipids profile 24.

Possible Ligands for SPF

Since SPF stimulates microsomal squalene monooxygenase and appears to promote intermembrane transfer of squalene, it was postulated early on that SPF is a squalene transfer protein. However, multiple attempts to show squalene binding were unsuccessful, or showed only weak affinity, likely not sufficient for direct transfer of squalene 27.

The sequence similarity of SPF to the yeast phosphatidylinositol transfer protein Sec14p suggested that phosphatidylinositol might be a ligand for SPF. *Saccharomyces cerevisiae* Sec14p binds phosphatidylinositol (PI) and phosphatidylcholine. In fact, SPF binds to PI with affinity of ~216 nM 27, which is the greatest reported binding affinity of any ligand tested with this protein. Despite containing the Sec14 domain, the lipid-binding pocket of SPF is larger than that of Sec14p. That leaves a possibility that a larger lipid molecule, perhaps one of the phosphorylated forms of phosphatidylinositol, might be a physiological ligand for SPF.

SPF and Vitamin E

Using radiolabeled α-tocopherol as ‘bait’, a 46-kD protein was isolated from the cytosol of bovine liver in 1999. It was named α-tocopherol-associated protein (TAP) 28. As noted earlier, subsequently it was found that TAP is identical to SPF 13. Since then, many reports showing the ability of TAP to bind various forms of vitamin E have been
published\textsuperscript{11; 27; 29-31}. TAP is closely related to $\alpha$-tocopherol transfer protein (TTP), the principal tocopherol binding/transport protein in the cell [ ]. However, in TTP-null mice plasma alpha-tocopherol levels are low but levels of liver SPF are not altered, suggesting that SPF is not directly involved in $\alpha$-tocopherol homeostasis\textsuperscript{32}.

The term Vitamin E describes a family of tocols and tocotrienols, including $\alpha$-, $\beta$-, $\gamma$-, $\delta$-tocopherols, which can be distinguished by differently methylated chromanol headgroups. Tocotrienols can also have differently methylated headgroups, but in contrast to tocopherols, they have three unsaturated bonds in the phytol tail. The only naturally occurring stereoisomer of $\alpha$-tocopherol is RRR-$\alpha$-tocopherol (2R, 4'R, 8'R-$\alpha$-tocopherol). This is also the most important physiological form of Vitamin E\textsuperscript{33}. SPF/TAP was reported to bind to tocopherols, tocotrienols and tocoquinones with various affinities\textsuperscript{27; 29; 31; 33}. Among the tocols, the best binding affinity was reported with $\gamma$-tocopherol, followed by $\beta$-tocopherol and $\alpha$-tocopheryl quinone, an oxidized form of $\alpha$-tocopherol\textsuperscript{11; 27}. Binding affinity to tocotrienols was not determined.

Despite numerous efforts, it is still unclear what the physiological ligand for SPF is, a characteristic problem for Sec14 proteins. The possibility exists that all these molecules are ligands for SPF under different conditions, or in different cell types, and they may also result in different downstream effects\textsuperscript{19}.

\begin{figure}[!h]
\centering
\includegraphics[width=\textwidth]{tocopherol_tocotrienol.png}

\begin{itemize}
  \item $\alpha$-tocopherol ($R_1=CH_3$, $R_2=CH_3$)
  \item $\beta$-tocopherol ($R_1=CH_3$, $R_2=H$)
  \item $\gamma$-tocopherol ($R_1=H$, $R_2=CH_3$)
  \item $\delta$-tocopherol ($R_1=H$, $R_2=H$)
\end{itemize}

\textbf{Figure 1.5: Chemical structures of tocopherols and tocotrienols.}
Is SPF a transcriptional regulator?

As mentioned above, SPF was purified from bovine liver using α-tocopherol as a bait, and there are several reports of SPF binding to vitamin E; whether this link to vitamin E is relevant to the stimulatory effect on cholesterol synthesis is unclear. Interestingly, in COS-1 cells, SPF moved from the cytosol to the nucleus in response to α-tocopherol treatment, and α-tocopherol-dependent transcriptional activation was demonstrated, raising the question: is SPF a transcription factor? Indeed, α-tocopherol has been shown to regulate the transcription of various genes. α-Tocopherol downregulates transcription of the cholesterol scavenger receptors SR-A and CD36, and upregulates the expression of α-TTP protein, and thus may regulate its own homeostasis. In addition, α-tocotrienol and γ-tocotrienol were shown to inhibit cholesterol synthesis via post-transcriptional inhibition of HMG-CoA reductase with γ-tocotrienol being much more potent than α-tocotrienol. This effect was later shown to be due to the tocotrienol stimulation ubiquitination and degradation of HMG-CoA reductase. A general inhibitory effect of α-tocopherol on most cholesterologenic enzymes was described recently. The mechanism of this inhibition was attributed to the modulation of SREBP-2 processing, preventing the SREBP-2 mature form to reach the nucleus, in a manner analogous to that of sterols. In general it appears that actions of α-tocopherol are in opposition to those of SPF, which leads to the hypothesis that α-tocopherol binding to SPF, if it occurs, might prevent the stimulatory effect of SPF on cholesterol synthesis. However, a study from our laboratory failed to demonstrate an effect of α-tocopherol on the ability of SPF to stimulate squalene monooxygenase, and therefore a link between SPF, Vitamin E, and cholesterol synthesis, if it exists, remains to be elucidated.
A role for SPF in cancer

Epidemiologic studies indicated that vitamin E has a protective effect against prostate cancer \textsuperscript{39}. Since SPF was shown to be linked to vitamin E, the effect of SPF on prostate cancer was investigated. Indeed SPF, as α-tocopherol-associated protein (TAP), was shown to inhibit the growth of prostate cancer cells in cell culture \textsuperscript{40}. Two separate mechanisms were identified: First, facilitation of vitamin E uptake and mediation of its antiproliferative effect; and second, an effect independent of vitamin E, based on its suppression of PI3K/Akt signaling, a major survival pathway in prostate cancer \textsuperscript{40}. Immunohistochemical analysis of tissues from patients that underwent prostatectomy showed that expression of SPF was reduced in areas of malignancy compared with normal or benign areas. A significant reverse correlation was also found between SPF expression and aggressiveness in malignancy. Also, a high number of SPF-positive cells correlated with a lower incidence of recurrence after surgery. These interesting findings pointed to SPF as a predictor of cancer disease progression and recurrence \textsuperscript{41}.
INTRODUCTION

There are three homologous SPF genes in humans: hSPF1, hSPF2 and hSPF3. All three genes are located on chromosome 22. Protein and mRNA identity between these three SPFs is shown in Fig. 2.1.

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Figure 2.1: Human SPF and SPF-like proteins. The table displays the percent identity between these three SPFs at the mRNA, protein, and domain level.
The best characterized SPF protein to date is SPF1. Much less is known about the expression and function of the two other SPF-like proteins: SPF2 and SPF3. SPF1 was discovered in the liver cytosolic fraction as a factor that stimulated squalene monooxygenase. Many years later, when the mRNA sequence of SPF was reported, several studies were carried out to determine which tissues express this gene. It was reported that, in addition to liver, SPF transcripts could be detected in small intestine, brain, lung and skin; however, at that time the additional SPF-like genes had not been discovered, and so the possibility of multiple gene transcripts was not addressed. Later studies by another group identified prostate as a site of SPF1 expression, and Kempna et al. isolated cDNAs to all three SPF genes from human lung cDNA library and reported the expression of all three transcripts in liver, lung, whole blood and skin; expression of SPF2 was lowest of the three genes in these tissues. Later, another group confirmed expression of SPF2 in liver, but not in lung. Recently, brain and adrenal gland were identified as additional sites of all three SPF transcripts presence. Moreover, SPF3 was found in ovary, testis, epithelial duct cells of several glands and endothelial cells of small arteries.

The reported discrepancies in the tissue-specific expression of the SPF genes may result from the use of probes and primers that were not optimized to distinguish between these three closely related transcripts. Given the great degree of similarity between these transcripts, special attention should be put into the design of gene-specific probes that will detect only the transcript of interest, without crossreacting with transcripts from the homologous genes. To address this need and to unify the expression data for all three SPF genes, I designed gene-specific oligos for each SPF transcript, so as to optimize the signal detection and specificity and allow analysis of the expression pattern of all three genes in multiple tissues concurrently. Also, for the first time, the relative levels of each transcript were to be analyzed in selected tissues so as to gain insight into the relative abundance of each SPF transcript.
Experimental Rationale

There are three homologous SPF genes in human. There were several attempts to establish tissue-specific expression pattern for corresponding transcripts\textsuperscript{11; 12; 31}, however I felt they did not exhibit enough precautions to fully differentiate between these highly similar transcript sequences. To address these potential short-comings, the expression pattern of three SPF transcripts was assessed with use of highly specific probes designed for each SPF transcript. A broad range of tissues was screened concurrently so as to assess the tissue specific expression pattern of each of these genes. In addition, the relative levels of all SPF transcripts in selected tissues was determined by RT-PCR. We hypothesize that conclusions drawn from this expression data would be useful in designing further studies aimed at elucidating the role or roles of the various SPF genes in cellular and organismal physiology.

MATERIALS AND METHODS

Cloning of cDNAs to SPF2 and SPF3

Amplification: To amplify SPF2 and SPF3 coding sequences, sequence-specific primers were designed in such a way that they would incorporate an \textit{NdeI} restriction site at the start codon and an \textit{XhoI} restriction site immediately before the stop codon. For SPF2 the primers were 5’-\textit{catatgAGCGGCGAGTTGGAGAC-3’} and 5’-\textit{ctcgagGACAGGGGTAGCTCCTTATC-3’} and for SPF3 they were 5’-\textit{catatgAGCAGCCGAGTCGGGGAC-3’} and 5’-\textit{ctcgagCTGTGGTTGGGAGGAGGTCTC-3’}.

Incubation mixtures (50 µl) contained: 1 µg of human lung cDNA library (Clontech), SPF3 \textit{Ndel>} and SPF3 \textit{Xhoi<} primers (1 µM each), 2.5 U of \textit{Pfu} Turbo\textsuperscript{TM} DNA polymerase (Stratagene), dNTP mix (0.5 mM each), 1x \textit{Pfu} buffer, and water. PCR cycling parameters were: 5 minutes at 95°C for initial denaturation, followed by 35 cycles of: denaturation for 1 minute at 95°C, annealing for 1 minute at 64°C, extension at 72°C for
90 seconds, and final extension at 72°C for 10 minutes. The PCR product was analyzed by gel electrophoresis on a 1% agarose gel containing ethidium bromide for DNA visualization.

Subcloning into TOPO: the PCR product was subcloned into Zero Blunt®TOPO® vector (Invitrogen). The TOPO cloning reaction consisted of 4 µl of PCR product, 1 µl of Zero Blunt®TOPO® vector, and 1 µl of salt solution (0.2 M NaCl, 0.01 M MgCl₂) combined in a small tube and incubated for 7 minutes at room temperature. For transformation, 1-4 µl of the TOPO cloning reaction was added to TOP10 chemically competent E.coli cells, mixed gently, and incubated on ice for 15-30 minutes. The cells were heat-shocked for 45 seconds at 42°C and placed back on ice. LB media (250 µl) was added to the cells and the tube was shaken gently at 37°C for 1 hour. From 50-200 µl of each transformation was spread on a prewarmed selective agar plate (kanamycin for TOPO Blunt) and incubated overnight at 37°C. The next day 6-12 colonies were picked for analysis.

Analyzing Positive Clones: Colonies were cultured overnight in LB medium with kanamycin. Plasmid DNA was isolated by miniprep (Promega). Plasmids were analyzed by restriction analysis with restriction sites used for cloning (NdeI, XhoI) or EcoRI. The restriction digestion reaction was performed by mixing 1-2 µg of plasmid DNA with 2 units of NdeI and 2 units of XhoI in 1xNEBuffer #4 at total volume of 25 µl. Digestion reactions were incubated for 1-3 hours at 37°C and analyzed by gel electrophoresis on 1% agarose gels to visualize the size of the insert. The band containing the insert of expected size (1200 bp) was cut out of the gel, placed in a 1.5 ml tube with a filter, and purified in a microfuge for 5 minutes at 5000 x g.

Cloning into bacterial expression vector: The pTYB2 vector (New England Biolabs) was digested with NdeI and XhoI restriction enzymes and ligation of the insert into the vector was performed as follows. Purified insert and digested vector were combined together with an excess of the insert at a molar ratio of 3:1 in a 20-µl reaction containing 0.1-1 U of T4 DNA ligase (Invitrogen) and 1x ligase buffer. The mixture was incubated at room temperature for 1 hour followed by transformation and analysis of
positive clones as described above. Positive clones were sent to a commercial firm (Davis Sequencing, Davis, CA) for sequencing.

Cloning into mammalian expression vector pc3.1DNA(-): The complete coding sequence of the SPF3 cDNA was amplified by *Pfu* polymerase, as described earlier, with the use of primers incorporating *Nde*I and *Xho*I restriction sites at the N- and C-termini. The reverse primer additionally incorporated a stop codon prior to the *Xho*I restriction site. The amplified sequence was ligated into the Zero Blunt TOPO vector, plasmid DNA purified, and the insert was released with *Nhe*I and *Xho*I restriction enzymes. The insert was purified by agarose gel electrophoresis and ligated into pc3.1DNA(-) vector (Invitrogen). Ligation conditions were as described above.

**Multiple Tissue cDNA Panels**

Gene-specific primers were selected by visual inspection, comparing the three SPF sequences for regions of divergence. (These primers are shown in Table 2.1.) For optimization the primers were first tested on each of the three SPF cDNAs. The vectors containing these sequences were: pTYB4SPF1 and pTYB2SPF3 for the full-length SPF1 and SPF3 cDNAs, and ZeroBluntTOPOSPF2600 for the 600-bp fragment of the SPF2 cDNA. Various polymerases and temperature cycling conditions were tested until the optimal result was obtained with *Taq* Polymerase, using a manual ‘hot start’ protocol. In this protocol the PCR Master Mix was prepared without *Taq* Polymerase, the reactions were mixed with 5 μl of the cDNA sample in total volume of 50 μl and heated to 75°C for 1 minute, and then *Taq* polymerase was added and thermal cycling started. Cycling conditions were: 95°C for 3 minutes, followed by 30 cycles of: 95°C for 45 seconds, 55°C for 30 seconds, 72°C for 90 seconds, followed by 10 minute extension at 72°C. PCR products were then analyzed by gel electrophoresis with staining with ethidium bromide.
Multiple Tissue Expression Arrays

The oligonucleotide probes used in this study were selected from the proximal 3’ untranslated region of each mRNA. Each probe was fifty nucleotides long and had three biotin moieties at the 5’ end. The sequences of the probes are shown below in Table 2.3. Hybridization to the MTE blot was performed according to the manufacturer’s instructions (BD Biosciences). Sheared salmon testis DNA was denatured by heating at 90-100°C for 5 min, then quickly chilling on ice. Hybridization solution was prepared by mixing BD ExpressHyb Solution (BD Biosciences) with heat-denatured sheared salmon testis DNA. The membrane was prehybridized in hybridization solution with continuous agitation for 30 minutes at 42°C. Each oligonucleotide probe was denatured as described above for salmon testis DNA and mixed with hybridization solution, at a final concentration of 150 ng/ml. Hybridization was carried out overnight with continuous agitation at the temperature indicated in Table 2.1. The membrane was washed 5 times for 20 minutes with Wash Solution 1 (0.3 M NaCl, 30 mM Na\textsubscript{3}Citrate•2H\textsubscript{2}O, 1% SDS) at the hybridization temperature. The membrane was incubated with constant agitation for 8 minutes in Blocking Buffer (SpotLight™ Hybridization & Detection Kit, BD Biosciences), then Streptavidin-HRP conjugate was added and incubated for 15 minutes. The membrane was washed 4 times for 2 minutes with 1x Wash Buffer (BD Biosciences, Clontech), transferred to another container and incubated for 5 minutes with Substrate Equilibration Buffer (BD Biosciences, Clontech), after which the membrane was incubated with the mix of Luminol/Enhancer Solution and the Stable Peroxide Solution (BD Biosciences, Clontech) for 5 minutes and exposed in the Kodak 1D™ Imager for 30-60 seconds. The picture was analyzed with the Kodak 1D™ Imager’s software; dots were analyzed as Region of Interest areas (ROIs) and their net intensities were graphed. Each membrane was blotted with one probe only. The membrane could not be rebotted because stripping was not efficient in removing the probe, despite moderating the stripping conditions.
Table 2.1: Oligonucleotide probes used for hybridization to MTE arrays with their hybridization temperatures. X=biotin.

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RT-PCR analysis of relative expression levels

Construction of a Standard-Curve Template: For the RT-PCR studies it was necessary to construct a template containing the three corresponding cDNA segments of each SPF mRNA. This template would then be used to generate a standard curve for analysis of expression of the three SPF genes in each tissue. Short ~200-bp segments from SPF1 and SPF3 transcripts were amplified by PCR from a liver single-stranded cDNA library created from liver total RNA by reverse transcription. The primers used to select these segments were: 5’-CTCACCTGCACTGATCCTGGCA-3’ and 5’-GGGAGGCCTGCTATAGG-3’ for SPF1, 5’-gttacATGGGAACCTCACCTGCA-3’ and 5’-gggccGAGGAGTCTGAGAA-3’ for SPF2, 5’-acagtAGCCCTACCTGAGGCTGGG-3’ and 5’-actagGTCAGCAGGAGGTGGG-3’ for SPF3. These 3 cDNA segments were labeled Real1, Real2 and Real3, subcloned into the ZeroBluntTOPO or TOPOTA vector and then cloned into pTYB11 vector to serve as a template for RT-PCR (see Fig. 2.2, below).
Figure 2.2: Cloning scheme for the pTYB11 Real1, Real2, and Real3 templates.
Reverse Transcriptase Reaction: First-strand cDNA synthesis was performed using SuperScript II Reverse Transcriptase (Invitrogen). In a nuclease-free tube, 200 ng of random primers, 1 ng-5 µg total RNA (Clontech), and 1 µl of dNTP mix (10 mM each) were combined in total volume of 12 µl in diethylpyrocarbonate-treated water. The mixture was heated to 65°C for 5 minutes, quickly chilled on ice, briefly centrifuged, and 4 µl of 5x First-Strand Buffer, 2 µl of 0.1M DTT, 1 µl RNaseOUT (Invitrogen), and 200 units of SuperScript II Reverse Transcriptase were added. The reaction was incubated at 25°C for 10 min, followed by 50 min at 42°C and then inactivated by incubation at 70 °C for 15 minutes. Synthesized single-stranded cDNA (sscDNA) was quantified, aliquoted and stored in -80°C.

Real-Time PCR: Gene-specific primers for real-time PCR were designed by visual inspection or by using ABI Prism Primer Express software (Applied Biosystems). For SPF1 they were: 5’-TGCAGTGATCCTGGCATCTATG-3’ and 5’-TGAGGCTTTGCTGGAAGCAG-3’, yielding an amplicon of 108 bp; for SPF2: 5’-TCAGAGGCCGCGTCTATGT-3’ and 5’-GTGAGCTCCTTATCATATTTCTGCAT-3’ (amplicon of 129 bp); for SPF3: 5’-AGCCTCACCTGCCTCCAGG-3’ and 5’-CATCGCCTTGAGACTCTGCAG-3’ (amplicon of 146 bp). Each 20-µl PCR reaction contained 0.2 μg of single-stranded cDNA or template, gene-specific primers (200 nM each), 3 mM MgCl₂, dNTP mix (200 µM each), 1.25 unit of AmpliTaq Gold (Applied Biosystems), 0.2 units of AmpEraseUNG to prevent product carryover, and 1x SYBR Green PCR buffer. A standard curve was created for each pair of gene-specific primers with 10-fold dilutions of template (pTYB11 Real1, Real2, and Real3). Cycling conditions were: 2 minutes at 50°C to activate AmpEraseUNG, initial denaturation for 10 minutes at 95°C, followed by 40 cycles of: denaturation for 15 seconds at 95°C and 1 minute of annealing/extension at 58°C. In order to detect nonspecific amplification, dissociation curves were established for each PCR product or PCR products were analyzed by gel electrophoresis.
RESULTS AND DISCUSSION

Sequence Alignments

Measurement of mRNA levels was chosen as the first means for assessing expression of the three human SPF genes. First, the mRNA sequences of three SPFs were aligned with use of multiple sequence alignment tool AlignX (VectorNTI, Invitrogen). This alignment revealed the regions of highest similarity and those that were less conserved (Fig. 2.3). SPF1 and SPF2 mRNAs were 66% identical, SPF1 and SPF3 mRNAs were 59% identical, while SPF2 and SPF3 mRNAs were 61% identical.
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<td>CGCGGGAACAAUGAGCG</td>
<td>GCAGCUUUAGCAACAG</td>
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<tr>
<td>Consensus (2129)</td>
<td>GCAACAGCACGCG</td>
<td>GCCAGGCGC</td>
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<th>NM_149767 (1926)</th>
<th>NM_149777 (1641)</th>
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<tr>
<td>Consensus (2241)</td>
<td>GCAACAGCACGCG</td>
<td>GCCAGGCGC</td>
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<th>NM_149777 (1641)</th>
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<tbody>
<tr>
<td>ACCUGCGGAGGAGGAG</td>
<td>CGCGGGAACAAUGAGCG</td>
<td>GCAGCUUUAGCAACAG</td>
</tr>
<tr>
<td>Consensus (2297)</td>
<td>GCAACAGCACGCG</td>
<td>GCCAGGCGC</td>
</tr>
</tbody>
</table>
Figure 2.3: Multiple sequence alignment of the three human SPF mRNAs (sections 1-52). NM_012429, human SPF1 mRNA, 2752 bp; NM_174975, human SPF2 mRNA, 2065 bp; NM_144977, human SPF3 mRNA, 2500 bp. This multiple sequence alignment was created with use of the sequence comparison algorithm AlignX of Vector NTI Advance10 (Invitrogen). The SPF gene thought to correspond to the most widely characterized SPF is NM_012429. Sections 11, 13, 14 and 18 are annotated to show the primers used for
the Multiple Tissue cDNA Arrays, as described below. Sections 25 and 26 are annotated to show the oligonucleotide probes used for hybridization to Multiple Tissue Expression arrays, as described below.

**Cloning of SPF2 and SPF3 cDNAs**

The coding sequence for SPF1 was already available in our lab and had been cloned into the pTYB4 vector (New England Biolabs, Inc.). I was unable to amplify the full-length cDNA for SPF2 from human liver or small intestine cDNA libraries, despite a variety of reaction conditions and multiple primer pairs tested. Only a ~600 bp fragment (302-882 bp of the corresponding mRNA) was able to be amplified from a human small intestine cDNA library, and it was subsequently cloned into the Zero Blunt TOPO vector.

The SPF3 cDNA was amplified from a human lung cDNA library with sequence-specific primers derived from the published transcript sequence (NM_174977). The sequence of the polymerase chain reaction (PCR) product was determined and confirmed to be identical with SPF3. This PCR product was cloned into the Zero Blunt TOPO shuttle vector (Invitrogen), plasmid DNA was isolated, the insert was released with NdeI and XhoI, purified, and cloned into the bacterial expression vector, pTYB2 (New England Biolabs, Inc.).

**Multiple Tissue cDNA Panels**

The expression of the three SPF genes was first analyzed by PCR using gene-specific primers on a set of first-strand cDNAs generated from 8 human tissues (MTC I panel, Clontech). The tissues analyzed were: heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas. Gene-specific primers for each sequence were designed and the specificity of each primer-pair was tested by PCR on cDNA templates for each SPF cDNA.
available in the laboratory. Multiple primer-pairs were tested and the best specificity was obtained with the following primers, as shown in Table 2.2.

Table 2.2: Gene-specific primers used in the MTC-PCR experiments.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
<th>PCR product size</th>
<th>Annotation in Fig 2.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPF1</td>
<td>Fw: 5’-CCACCAGACCACAAGTTG-3’&lt;br&gt;Rv: 5’-GGCTTTAACAACAAAAAGACG-3’</td>
<td>~200 bp</td>
<td>in red in Sections 11&amp;14</td>
</tr>
<tr>
<td>SPF2</td>
<td>Fw: 5’-CAGAGACCCTGAAGTTCATG-3’&lt;br&gt;Rv: 5’-CAGGTACATGGACTTGGGG-3’</td>
<td>~260 bp</td>
<td>in green in Sections 13/14 &amp; 18</td>
</tr>
<tr>
<td>SPF3</td>
<td>Fw: 5’-GTTGCATGAGTGTGAGCTGC-3’&lt;br&gt;Rv: 5’-CAGGTAGTAGCTTGGGC-3’</td>
<td>~420 bp</td>
<td>in blue in Sections 11&amp;18</td>
</tr>
</tbody>
</table>

These primer-pairs were highly specific for their cognate cDNAs, as shown in Fig. 2.4. With the confirmed specificity of these gene-specific primers, the expression pattern of the three SPF genes in tissues from a Multiple Tissue cDNA panel (MTC) was examined (Fig. 2.5). SPF1 appeared to be expressed in all tissues, as, despite the smear, a band of ~200bp can be seen in placenta, pancreas, brain, liver and kidney. A band is less evident in lung, heart and muscle, but from this experiment expression cannot be definitively ruled out. Improved PCR results or another approach would be needed to rule out this
possibility. For SPF2 no distinct band of ~260 bp was observed in any tissue. However, the smearing pattern on the gel appears to be different for different tissues and this may be due to some nonspecific binding of primers, or there might be some product which is not visible due to the smearing. With SPF3 a clear result indicating no expression in any of the tissues, except for pancreas, was obtained. In general, the inability to obtain a clean PCR product in these tissues with these primers prevented the unambiguous interpretation of these results, and therefore the outcome of this experimental approach was deemed unsatisfactory.

![Figure 2.4: Specificity of the SPF primers.](image)

_Gel electrophoresis of the PCR products obtained with A, SPF1-specific primers; B, SPF2-specific primers; C, SPF3-specific primers. In each panel the lanes are as follows: lane 1, 500-bp DNA ladder; lane 2, PCR product with SPF1 as a template; lane 3, PCR product with SPF2 as a template; lane 4, PCR product with SPF3 as a template._
Gel electrophoresis of the PCR products obtained with each set of primers is as follows: A, lane 1, 500-bp DNA ladder; lane 2, glyceraldehyde-3-phosphate dehydrogenase (G3PDH) PCR product (1000 bp) from control cDNA provided by the manufacturer; lanes 3-11, PCR products obtained with SPF1 primers on different tissue cDNA templates; lanes 12-20, PCR products obtained with SPF2 primers. The DNA templates are indicated above each lane. B, lane 1, 500-bp DNA ladder; lane 2, empty; lanes 3-10, PCR products obtained with SPF3 primers on different tissue cDNA templates, as indicated above the figure.

Multiple Tissue Expression arrays

Because of the inconclusive results obtained with the MTC-PCR approach described above, hybridization of gene-specific probes to Multiple Tissue Expression (MTE) arrays was chosen as an alternative approach to assess tissue expression patterns. MTE arrays consist of a nylon membrane with immobilized polyA⁺ RNA from 75 different human tissues (Table 2.3), allowing the screening of a broad range of tissues in a single experiment. Moreover, the probes for this experiment were designed to the 3′-untranslated regions of the mRNAs, which share much less identity between SPF genes.
than do the coding regions. Therefore, these probes were expected to overcome the problems encountered with primer specificity in the earlier MTC-PCR experiments. The probe sequences used in these experiments are indicated in Fig 2.3 in Sections 25 and 26, and are listed in Table 2.2. Probes were hybridized to the MTE arrays as described in Materials and Methods (see below). After hybridization the signal from the mRNA dots was analyzed and net intensities were determined. As shown in Figure 2.6, for the SPF1 transcript the strongest signal was obtained in mammary gland (F9), stomach (B5) and liver (A9). The signal from trachea (H7), prostate (E8) and testis (F8) was weaker but noticeable. There was no distinguishable signal from either SPF2 or SPF3 in this experiment (Figures 2.7 and 2.8).

High expression of SPF1 in liver was not surprising, as it is a main site of cholesterol biosynthesis. Similarly, prostate and testis are also sites of cholesterol and sex hormone synthesis. Moreover, high expression of SPF1 in liver and prostate has been demonstrated by others. Mammary gland, especially during lactation, is a site of intensive lipid uptake, de novo biosynthesis, transport and excretion; the expression of SPF1 in this tissue is consistent with a role for this protein in cholesterol synthesis and/or processing. The basis for expression of SPF in trachea and stomach is less evident, but suggests that SPF may have additional functions not yet recognized. It should be noted that polyA+ RNA was prepared from the whole organs without distinguishing specific cell-types within the organ, and it is possible that SPF is important in particular cell-types within an organ. Indeed, there are reports demonstrating the presence of SPF in endothelial cells of various organs or tissues, consistent with a model in which SPF serves as a transporter protein and may be involved in lipid absorption or secretion events. Further investigation on cell-type specificity of expression within various tissues and cell types will be needed to elucidate such functions.

MTE arrays were normalized to the expression levels of eight different housekeeping genes to make possible the quantification of signal and conclusions regarding relative transcript abundance in different tissues. For this reason different amounts of mRNA
(from 37 to 1127 ng per dot) were loaded on each blot to account for differences in transcriptional activity of different tissues (Table in Fig 2.3). Graphs in Figures 2.6-8 display net signal intensity obtained from each tissue after hybridization with each probe. The results displayed are one-time measurements, as the probes could not be stripped from the blots effectively. Due to the high cost of each blot, the hybridizations were performed only once for each probe. For this reason the results were not treated as quantitative and further conclusions on relative transcript abundance in different tissues cannot be made from these data. Similarly, the results from each blot cannot be compared to each other as the blotting was performed at different times with different probes and there is no control that would allow for comparison between blots. Therefore the relative levels of each transcript in a single tissue cannot be determined from these data and another approach had to be undertaken to answer this question.
Figure 2.6: Human SPF1 tissue specific expression. Multiple Tissue Expression (MTE) Array probed for presence of SPF1 gene.
Figure 2.7: Human SPF2 tissue specific expression. Multiple Tissue Expression (MTE) Array probed for presence of SPF2 gene.
Figure 2.8: Human SPF3 tissue specific expression. Multiple Tissue Expression (MTE) Array probed for presence of SPF3 gene.
Table 2.3: Positioning and amount of all polyA+ RNAs loaded on Multiple Tissue Expression blots.

<table>
<thead>
<tr>
<th>Position</th>
<th>Tissue</th>
<th>ng/dot</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>whole brain</td>
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</tr>
<tr>
<td>B1</td>
<td>cerebral cortex</td>
<td>204</td>
</tr>
<tr>
<td>C1</td>
<td>frontal lobe</td>
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</tr>
<tr>
<td>D1</td>
<td>parietal lobe</td>
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</tr>
<tr>
<td>E1</td>
<td>occipital lobe</td>
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</tr>
<tr>
<td>F1</td>
<td>temporal lobe</td>
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<tr>
<td>G1</td>
<td>postcentral gyrus of cerebral cortex</td>
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</tr>
<tr>
<td>H1</td>
<td>pons</td>
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<td>F2</td>
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<tr>
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<td>medulla oblongata</td>
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<tr>
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<td>G4</td>
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<tr>
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<tr>
<td>Column</td>
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</tr>
<tr>
<td>Column</td>
<td>A8 lung</td>
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<tr>
<td>Column</td>
<td>B8 placenta</td>
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<td>Column</td>
<td>C8 bladder</td>
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<td>D8 uterus</td>
<td>158</td>
</tr>
<tr>
<td>Column</td>
<td>E8 prostate</td>
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<tr>
<td>Column</td>
<td>F8 testis</td>
<td>386</td>
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<tr>
<td>Column</td>
<td>G8 ovary</td>
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<tr>
<td>Column</td>
<td>B9 pancreas</td>
<td>1127</td>
</tr>
<tr>
<td>Column</td>
<td>C9 adrenal gland</td>
<td>245</td>
</tr>
<tr>
<td>Column</td>
<td>D9 thyroid gland</td>
<td>188</td>
</tr>
<tr>
<td>Column</td>
<td>E9 salivary gland</td>
<td>548</td>
</tr>
<tr>
<td>Column</td>
<td>F9 mammary gland</td>
<td>101</td>
</tr>
<tr>
<td>Column</td>
<td>A10 promyelotic leukemia, HL-60</td>
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</tr>
<tr>
<td>Column</td>
<td>B10 Hela S3</td>
<td>95</td>
</tr>
<tr>
<td>Column</td>
<td>C10 K-562, chronic myelogenous leukemia</td>
<td>69</td>
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<tr>
<td>Column</td>
<td>D10 MOLT-4, lymphoblastic leukemia</td>
<td>42</td>
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<tr>
<td>Column</td>
<td>E10 Raji, Burkitt's lymphoma</td>
<td>54</td>
</tr>
<tr>
<td>Column</td>
<td>F10 Daudi, Burkitt's lymphoma</td>
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<td>Column</td>
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<td>Column</td>
<td>H10 A549, lung carcinoma</td>
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<td>A11 fetal brain</td>
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</tr>
<tr>
<td>Column</td>
<td>B11 fetal heart</td>
<td>238</td>
</tr>
<tr>
<td>Column</td>
<td>C11 fetal kidney</td>
<td>284</td>
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</tbody>
</table>
Real-Time Polymerase Chain Reaction (RT-PCR) with the use of a standard curve was employed to assess the relative levels of expression of three SPF transcripts in selected tissues. First, the template for the standard curve was created by cloning a short segment of each SPF cDNA into the single vector (Fig. 2.9). A series of template dilutions was prepared and the standard curve for each SPF gene was generated (Fig. 2.10).
Figure 2.9: Template for RT-PCR Standard Curve. A segment of each SPF cDNA was cloned into the pTYB11 vector.

Figure 2.10: Standard curves generated for each SPF separately from the same template.

Gene-specific primers were designed for each transcript and the specificity of these primer pairs was confirmed by standard PCR (Fig.2.11).
Figure 2.11: Specificity of SPF primers used for RT-PCR. Gel electrophoresis of the PCR products obtained with SPF1-specific primers, SPF2-specific primers, SPF3-specific primers on SPF1, SPF2 and SPF3 templates; NTC- no template control (water). For each pair of primers only a band for specific template was obtained, indicating no crossreactivity with the other two SPFs.

The expression of each SPF mRNA transcript relative to the other two SPF transcripts was determined with RNA from liver, stomach, and mammary gland; these tissues were selected because the MTE blots indicated that all three SPF transcripts were present in these tissues. First, total RNA was reverse-transcribed to a single-stranded cDNA template and the expression of each SPF transcript was measured by real-time PCR with gene-specific primers. The quantity of each transcript in these three tissues then was determined with use of the standard curve. As anticipated from the MTE hybridization results, the SPF1 transcript was most abundantly expressed in these tissues (Fig. 2.12).
In liver, SPF1 mRNA was ~20 fold higher than SPF3 mRNA, followed by very low levels of SPF2 mRNA. Liver was the only tissue where SPF2 mRNA was detectable. In stomach and mammary gland the trend was similar, with SPF1 mRNA levels higher than SPF3 levels by ~5 fold in stomach and ~70 fold in mammary gland (Fig. 2.11).

Summarizing, SPF expression was most evident in liver, stomach, and mammary tissue, with levels in liver being at least 50-fold higher than those found in other tissues. My
studies further show that, of the three SPF transcripts, SPF1 is the most abundant, followed by approximately 20-fold lower expression of SPF3 and negligible expression of SPF2. Although I had hoped to uncover differential expression of these genes in various tissues suggestive of functional differences, the expression patterns did not support this hypothesis. In all tissues where SPF was present, SPF1 expression dominated, suggesting that SPF2 and SPF3 were either non-functional, of marginal importance, or were expressed only in specific conditions or developmental stages. Further studies will be needed to identify any such conditions.
Chapter 3: ABILITY OF THE SPF3 PROTEIN TO STIMULATE CHOLESTEROL SYNTHESIS

INTRODUCTION

Homology is defined as similarity between two sequences or structures that is attributed to their shared evolutionary history. There are two types of homologous genes: orthologs and paralogs. Homologous sequences are referred to as orthologs if the point of divergence was a speciation event. Otherwise they are paralogs of one another, which is the case for the three human SPF genes. A common paradigm in proteomics is that structure defines function. Functional assignment is often made based on sequence or structural similarity; i.e., if a hypothetical protein is similar in sequence or structure to a known protein, it is often provisionally assigned that same function. It is thought that orthologs are more likely than paralogs to retain the same function, because the events that give rise to paralogs, such as gene duplication and horizontal gene transfer, provide opportunity for diversification of function through the relaxation of selective pressure 44; 45.

Experimental Rationale

Although it is clear that SPF1 can stimulate squalene monooxygenase in vitro and cholesterol synthesis when expressed in cultured hepatoma cells 12, it is not known if SPF2 and SPF3 possess the same stimulatory properties. Based on the high degree of sequence similarity between these gene sequences, I hypothesized that SPF2 and SPF3 might also stimulate cholesterol synthesis. However, as noted above, homology does not necessarily imply functional equivalence. Therefore, this hypothesis should be
tested experimentally. To do so I chose microsomal assays and hepatoma cell culture as experimental test systems.
MATERIALS AND METHODS

Recombinant Protein Expression and Purification

Recombinant protein was purified with use of the IMPACT (Intein Mediated Purification with an Affinity Chitin-binding Tag) protein purification system (New England Biolabs). The pTYB2 vector used for cloning is a fusion vector in which the C-terminus of a cloned protein is fused to the intein tag followed by chitin binding domain. The pTYB2 vector containing the SPF3 coding sequence (pTYB2 SPF3) was transformed into E.coli ER2566 competent cells carrying T7 RNA polymerase gene. Cells were grown at 37°C in 4 liters of LB medium containing 100 µg/ml ampicillin. When the OD₆₀₀ of the culture reached 0.6-0.8, protein expression was induced with IPTG at a final concentration of 0.5 mM. Cells were incubated overnight at constant agitation at room temperature. On the next day the cells were harvested by centrifugation at 5,000 x g for 10 minutes at 4°C. The cell pellet was resuspended in 100 ml of ice-cold Column Buffer (20 mM Tris-HCl, 500 mM NaCl, 1 mM EDTA, pH 8.0). Cells were divided into 20-ml portions and lysed by sonication on ice with 10-second bursts for a total of 5 minutes. A cleared lysate was obtained by centrifugation at 10,000 x g for 1 hour at 4°C. To reduce viscosity, lysates were treated with DNase for 30 minutes on ice. Cleared lysates were loaded onto the chitin column at 0.5 ml/min at 4°C. The column was washed with 10 bed-volumes of Column Buffer at 2 ml/min. On-column self-cleavage of the chitin-bound intein tag was induced by incubating column overnight in Column Buffer containing 50 mM β-mercaptoethanol and eluting the next day with Column Buffer. Eluted protein was then concentrated, the protein concentration was measured by the Bradford assay and its quality and purity was checked by SDS-gel electrophoresis, followed by Coomassie blue staining. Protein was aliquoted and stored at -80°C.
Squalene monooxygenase assay

Preparation of the liver microsomal fraction: Rats were killed by decapitation, the liver excised, and microsomes were prepared by standard procedures. In brief, the liver was minced thoroughly with scissors, transferred into 2 volumes of ice-cold homogenization buffer (100 mM Tris-HCl buffer at pH 7.4, containing 0.1 mM EDTA) and homogenized using a Potter-Elvehan homogenizer. All subsequent steps were carried out at 4°C. The samples were centrifuged at 10,000 x g for 20 min, and the pellet was discarded and the supernate was centrifuged at 100,000 x g for 60 min. The upper, lipid layer was removed and the cytosolic supernatant fraction collected. The microsomal pellet was resuspended with ice-cold homogenization buffer and recovered by 100,000 x g centrifugation for 60 min. The microsomal fraction was resuspended at ~20 mg of protein/ml in homogenization buffer and the cytosolic fraction (100,000 x g supernate) diluted to ~20 mg of protein/ml. The protein content was determined by Bradford assay with Coomassie Plus Reagent (Pierce). Aliquots were stored at -80°C.

Squalene Monooxygenase Assay: Microsomal squalene monooxygenase activity was determined with rat liver microsomes (400 µg/incubation) in a final volume of 0.2 ml as described previously. Radiolabeled 14C-squalene was synthesized by SRI International (Menlo Park, CA) and used at 7 mCi/mmol. In brief, 200 µg of microsomal protein, 30 µM FAD, 40 µM [14C]squalene, 10 µg of phosphatidylglycerol, and 0.3 mM AMO 1618 (Calbiochem) to inhibit oxidosqualene cyclase in 200 µl of 20 mM Tris-HCl buffer, pH 7.4, with 1 mM EDTA. Reactions were started by the addition of 1 mM of NADPH and placed in a 37°C water bath. After 1 h the incubation volume was brought to 1 ml with water and the reactions were stopped by the addition of 0.5 ml of 10% KOH in methanol. The tubes were capped, lipids were saponified at 80 °C for 1 h and then extracted with 3 ml of petroleum ether. The solvent layer was removed by centrifugal evaporation, the lipids resuspended in 50 µl of petroleum ether and spotted onto silica thin-layer plates. Lipids were fractionated with 5% ethyl acetate in hexane, visualized with iodine vapor stain, and quantified by electronic autoradiography (Packard Instant Imager). The ability
of SPF to stimulate squalene monooxygenase was determined by adding 0.8 µg of purified recombinant protein, unless indicated otherwise. Triton X-100 at 0.1% was used as a reference for maximal activation of microsomal squalene monooxygenase.

Cell culture

McARH7777 rat hepatoma cells were cultured in Dulbecco's Modified Eagle Medium (Gibco) supplemented with 1x Penicillin-Streptomycin mixture (Invitrogen) and 10% Fetal Bovine Serum (Invitrogen).

Transfections

The day before transfection, cells were plated onto glass coverslips at the density 5x10^4 cells per cm^2. On the next day plasmid DNA was transfected into cells with use of Fugene 6 (Roche Diagnostics). Plasmid DNA was mixed with Fugene 6 reagent in serum-free medium at a ratio of 1 µg:5 µl, and complexes were allowed to form for 30 min at room temperature. The medium on the cells was replaced with antibiotic-free medium with 10% FBS and the transfection mixture was added dropwise to the cells. Cells were assayed 48 hours post-transfection.

Cholesterol synthesis assay

Cholesterol synthesis was measured 48 hours post transfection as follows. The media was replaced and ^14C-acetate at 1 µCi/35 mm well was added and cells were incubated for 3 hours at 37°C. The media was removed, the cells were washed with PBS, and 1 ml of water was added and the cells were released by scraping. Cells were lysed by sonication in a water bath for 1 minute, after which 3 ml of a chloroform:methanol (2:1) mixture was added to each tube and the mixture was vortexed vigorously 5-10 times.
The lower fraction containing lipids was transferred into a new tube and the solvent was evaporated under liquid nitrogen. Samples were resuspended in 50 µl of a chloroform:methanol mixture and spotted onto silica thin-layer plates. Lipids were fractionated with petroleum ether:ethyl ether:acetic acid (60:40:1), cholesterol was identified by co-chromatography of authentic standard visualized by iodine-vapor staining, and quantified by electronic autoradiography (Packard Instant Imager).
RESULTS AND DISCUSSION

Expression and purification of human recombinant SPF1 and SPF3 proteins

Following the cloning of SPF3 into pTYB2 vector, as described in Chapter 2, the SPF1 and SPF3 proteins were expressed in *E.coli* strain ER2566 and purified following the protocol for intein-fusion proteins with the IMPACT system (New England Biolabs, Inc.). Because a full-length SPF2 cDNA could not be cloned, it was not possible to generate and test this putative protein. The pTYB vectors are designed specifically for purification with the IMPACT system, which attaches a self-splicing element (intein) followed by a chitin binding domain to the carboxy-terminus of the expressed protein, which allows for binding to a chitin affinity column. The expressed protein is then released with β-mercaptoethanol, which promotes intein self-splicing and release of protein. The cleaved, purified protein retains four extra amino acids at the C-terminus: Leu-Glu-Pro-Gly. The concentration of recombinant proteins was measured with use of the BCA protein assay (Pierce) and the purity was analyzed by SDS electrophoresis followed by Coomassie Blue staining (Fig. 3.1).

![Figure 3.1: SDS-gel electrophoresis of recombinant SPF1 and SPF3 proteins.](image-url)
Stimulation of squalene monooxygenase activity by SPF3

The ability of the recombinant human SPF1 protein to stimulate squalene monooxygenase activity by approximately 2-fold in microsomes has been reported \(^\text{21}\). Maximal stimulation was achieved with 0.8 μg of recombinant protein in a 200-μl incubation, while higher concentrations were inhibitory \(^\text{8, 21, 46}\). Therefore, a range of concentrations was tested with the SPF3 recombinant protein (Fig. 3.2). Triton X-100, which is strongly stimulatory to this enzyme \textit{in vitro}, was included as a positive control. Consistent with previous reports, recombinant SPF1 protein stimulated the activity of squalene monooxygenase by ~2 fold. Statistical analysis by one-way ANOVA showed that this stimulation is statistically significant (p < 0.05). In contrast, SPF3 protein was unable to stimulate squalene monooxygenase activity across the range of concentrations tested. This raises the possibility that the recombinant SPF3 protein has other functions or needs other conditions, or cofactors not supplied in microsomal assay to stimulate squalene monooxygenase. To address this possibility, the ability of SPF3 to increase cholesterol synthesis in mammalian cell culture was tested.
Figure 3.2: Squalene monooxygenase activity in the presence of recombinant SPF1 and SPF3. Radiolabeled 2,3-oxidosqualene formation from squalene was visualized and measured by electronic autoradiography (Packard Instant Imager). A) Radiolabeled 2,3-oxidosqualene visualized on a thin-layer chromatography (TLC) plate. B) Quantitative analysis of radiolabeled 2,3-oxidosqualene formation.
Stimulation of cholesterol synthesis in rat hepatoma cells

Cloning

In order to express the SPF3 protein in rat hepatoma cells, its cDNA had to be cloned into the mammalian expression vector pc3.1DNA(-) (Invitrogen). The SPF1 cDNA had been cloned into this vector earlier. The full-length SPF3 coding sequence was amplified from pTYB2SPF3 vector with the use of primers incorporating Ndel and Xhol restriction sites at the termini. The reverse primer incorporated a stop codon prior to the restriction site. The amplified sequence was ligated into the Zero Blunt TOPO vector, plasmid DNA purified, and the insert released with Nhel and Xhol restriction enzymes. The insert was then purified and ligated into the mammalian expression vector pc3.1DNA(-) vector.

Characterization of an antibody for quantitation of SPF3

To assess expression levels of SPF1 and SPF3 proteins after transfection into the hepatoma cells, an antibody detecting both proteins was needed. Rabbit polyclonal hSec14l2 antibody (GenWay) was previously tested on immunoblots and in immunofluorescence and showed a very good signal and the ability to detect the human, rat and mouse SPF proteins. To address the question of whether this SPF1 antibody also recognizes the SPF3 protein, 50-250 ng of each protein was fractionated by SDS-polyacrylamide gel electrophoresis, followed by electrophoretic transfer for immunoblotting. Band intensities were measured and plotted against the protein amount. It was found that rabbit hSec14l2 antibody (GenWay) detects both SPF1 and SPF3, although it has approximately 3-fold better sensitivity for SPF1 (Fig. 3.3).
Figure 3.3: Detection of SPF1 and SPF3 proteins with rabbit Sec14l2 antibody. A) Immunoblot of SPF1 and SPF3 recombinant proteins probed with rabbit polyclonal Sec14l2 antibody (GenWay, Inc). B) Semi-quantitative analysis of immunoblot.

HA epitope cloning

To achieve more comparable results, site-directed mutagenesis was employed to insert an hemagglutinin (HA) epitope at the C-terminus of SPF1 and SPF3 (this cloning procedure is described in Chapter 4). In subsequent experiments, rabbit polyclonal HA-probe (Y11) antibody (Santa Cruz Biotechnology, Inc.) was used to compare protein expression levels.
Cholesterol synthesis assay with SPF1 and SPF3

Rat hepatoma cells were transfected with SPF1, SPF3, or vector plasmid with Fugene6 transfection reagent (Roche) on day 0. On day 2 \textit{de novo} cholesterol synthesis from $^{14}$C-acetate was measured in a 3 h window. Results from 3 separate experiments are shown below (Fig. 3.4).

![Stimulation of cholesterol synthesis](image)

**Figure 3.4: Stimulation of cholesterol synthesis in rat hepatoma cells upon transfection with SPF1/3 expression plasmids.** White bars: control (lacZ-transfected cells); red bars, SPF1-transfected cells; blue bars, SPF3-transfected cells. Each value represents the mean ± S.E. of one experiment carried out in triplicate. Statistical analysis was performed with one-way ANOVA followed by Tukey’s Multiple Comparison Test; a single asterisk (*) indicates a significant difference from the control with $p<0.05$; a double asterisk (**) indicates a $p$ value $<0.01$.

As can be seen from this set of experiments, SPF3 was just as effective as SPF1 in stimulating cholesterol synthesis in transfected hepatoma cells. This result is in contrast to the lack of stimulation obtained with SPF3 in microsomal squalene monooxygenase.
assays (Fig. 3.2). To ensure that SPF3 expression in these cells was comparable to that of SPF1 in SPF1-transfected cells, mRNA and protein levels were determined in cells transfected with each plasmid. As shown in Fig. 3.5, mRNA and protein expression levels varied significantly between experiments, probably reflecting transfection efficiency. Within an experiment generally similar levels of expression were obtained for both proteins, suggesting that SPF1 and SPF3 proteins have similar synthesis/degradation profiles. Moreover, protein levels correlated reasonably well with mRNA levels within each experiment for each expression construct, again suggesting that the differences in protein levels between experiments largely reflected transfection efficiency rather than differences in transcription, translation, or mRNA or protein stability for either protein.
A) 

RNA amount [fold]

<table>
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<tr>
<th>SPF1</th>
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B) 

Protein amount [ratio HA tag signal/β-actin]

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C) 

Protein amount

RNA amount

R² = 0.6888
Figure 3.5: Expression levels of SPF1 and SPF3 in transfected rat hepatoma cells. Data are taken from three separate experiments. A) RNA levels assessed by RT-PCR; B) protein levels as determined from semiquantitative analysis of immunoblot images (shown below the graph). Proteins were probed with an antibody against the HA epitope (Y11); β actin was probed with a mouse anti β -actin antibody. Intensities of the bands were quantified with use of ImageJ software (NIH, MD) and expressed as the ratio of HA tag signal/β actin. C) Correlation of RNA and protein levels.

To assess the transfection efficiency of these plasmids, immunofluorescent staining of transfected cells was employed, using an antibody that recognizes the added HA epitope of each protein. Representative pictures are shown in Fig. 3.6. In these experiments transfection efficiencies were uniformly low (< 10%), but did not differ greatly between experiments or between plasmids. Moreover, the intensity of the staining did not differ between the plasmids, suggesting that both proteins were equally well expressed in these cells. In experiment #1 roughly ~1% of cells expressed SPF1 and ~3% expressed SPF3; in experiment #2 ~4% cells expressed SPF1 and ~8% expressed SPF3; in experiment #3 ~1% of cells expressed SPF1 and ~6% expressed SPF3. In general, higher transfection efficiencies yielded higher mRNA and protein expression levels, and largely correlated with the expression the corresponding SPF protein: Experiment 2 showed relatively high transfection efficiency for both plasmids, and the highest protein and mRNA levels as assessed by immunoblotting and RT-PCR, respectively; Experiment 3 showed the greatest difference in transfection efficiency between the two plasmids, and also the greatest difference in SPF protein expression levels.
Figure 3.6: Transfection efficiency of SPF1 and SPF3 expression plasmids as determined by immunofluorescent staining. Red, HA tag; blue, DAPI nuclear stain. Representative pictures from three separate experiments. In experiment #1 ~1% of cells expressed SPF1 and ~3% expressed SPF3; in experiment #2 ~4% cells expressed SPF1 and ~8% expressed SPF3; in experiment #3 ~1% of cells expressed SPF1 and ~6% expressed SPF3.

When the ability to stimulate cholesterol synthesis (data from Fig. 3.4) is expressed relative to the level of expression of each SPF protein (data from Fig. 3.5), the “specific activity” of each SPF can be determined, as shown in Fig. 3.7. From this analysis it appears that SPF1 is slightly more effective that SPF3 in its ability to activate this pathway, although the difference is not statistically significant.
Figure 3.7: Specific activity of SPF1 and SPF3 proteins to stimulate cholesterol synthesis. A) Data from three separate experiments; activity of SPF1 protein in each experiment was set as 100% in order to enable comparison with SPF3 “specific” activity. B) Average of the same data.

Several conclusions can be drawn from this data. The ability of SPF proteins to stimulate cholesterol synthesis appears quite robust; this conclusion is based on the 1.5- to 2-fold increase in radiolabel incorporation into cholesterol in cultures in which less than 10% of the cells are transfected with an expression plasmid. This suggests that the rate of
cholesterol synthesis in transfected cells is as much as 10-fold higher than that in untransfected cells. Secondly, the ability of SPF3 to stimulate cholesterol synthesis appears not to be dependent on a stimulation of squalene monooxygenase activity, as the recombinant protein was largely inactive in microsomal assays of squalene monooxygenase activity (Fig. 3.2). This is in marked contrast to SPF1, which is able to double the activity of this enzyme. This suggests that SPF3 acts at a second site in the cholesterologenic pathway, likely HMG-CoA reductase, the well-recognized regulatory step in cholesterol synthesis. It has been shown by this laboratory that SPF1 increases the activity of this enzyme in cell culture and in vitro. Because HMG-CoA reductase is typically the rate-limiting step in cholesterol synthesis, stimulation of this activity is almost certainly necessary to increase the overall rate of cholesterol synthesis in whole cells, with stimulation of squalene monooxygenase playing a secondary role. Thus, although SPF3 does not stimulate squalene monooxygenase activity, it may increase cholesterol synthesis by acting at HMG-CoA reductase to increase the overall activity of this pathway.

Finally, it is worth noting that SPF3 expression is approximately 20-fold lower than SPF1 in liver, based on the expression data presented in Chapter 2. Thus, in this highly cholesterologenic tissue SPF3 is not likely to play a significant role. It may assume greater importance in other tissues, such as stomach or mammary gland, or during development.
Chapter 4: INTRACELLULAR LOCALIZATION OF HUMAN SPF PROTEIN

INTRODUCTION

Supernatant Protein Factor (SPF) was originally purified from rat liver cytosol, and could not be detected in the microsomal fraction. Comparison to the database of known proteins revealed that SPF possesses a lipid binding domain (termed Sec14), and Golgi dynamics (GOLD) domain. This suggests that SPF might be an intracellular lipid transport protein and that it may interact with membrane components upon ligand binding or in response to certain stimuli.

In African green monkey kidney (Cos-7) cells, heterologously expressed SPF protein translocated into the nucleus upon the addition of α-tocopherol. It was further shown by luciferase reporter assay that SPF, upon addition of α-tocopherol to the cells, acts as a transcription factor. However, in cervical carcinoma (Hela) and mouse mast cells, heterologously expressed SPF protein localized to the cytosol and failed to move to the nucleus in response to α-tocopherol.

Recent studies by Zingg et al. have demonstrated that in mastocytoma (HMC-1) and lung carcinoma (A549) cells, endogenous SPF1 protein partially co-localized with the Golgi protein giantin. In Hela and adrenocortical (H295R) cells SPF co-localized with a mitochondrial marker. Localization of SPF1 to the Golgi suggested a role in this secretory pathway, while association with mitochondria was suggested to have specific function in the transport of lipid molecules (vitamin E, squalene, phospholipids) into and out of mitochondria. All these reports suggest that SPF localization events are cell-
type specific and may have different roles depending on cell type, ligand, or specific conditions.

Incubation of microsomes with the cytosolic fraction or recombinant SPF protein results in an increase of squalene monooxygenase activity. This effect was potentiated by addition of ATP to the cytosol, which suggested that secondary modification by phosphorylation may be necessary for full SPF activity. In support of this suggestion, addition of certain kinase inhibitors blocked the ATP-induced increase in SPF activity, at the same time narrowing the pool of kinases to protein kinase A (PKA) and C (PKC). A decrease in cytosolic SPF activity was also observed upon addition of various protein phosphatases, which were able to reduce SPF activity by ~50%. To extend these studies, recombinant SPF protein was preincubated with PKA or PKC in the presence of ATP prior to addition to the squalene monooxygenase assay. In both cases there was ~2 fold increase in SPF activity. The conclusion drawn from these studies was that SPF is activated by PKA and PKCδ by phosphorylation on one or more serine or threonine residues. The SPF sequence contains three consensus sequences for PKA and six for PKC. It was later found that rat SPF2 is also stimulated by PKA. Sequence alignment of rat and human SPF proteins revealed a single conserved PKA consensus site; substitution of a serine in this consensus site with alanine resulted in a mutated protein (SPF1S289A) which retained basal SPF activity in the microsomal assay but could no longer be activated by PKA or C. These in vitro results were later confirmed in a cell culture system. Cholesterol synthesis was elevated in rat hepatoma cells upon transfection with SPF and it was further increased when dibutyryl-cAMP, a PKA activator, was added. Total loss of SPF stimulation was observed upon addition of a cell permeable PKA inhibitor, 14-22 amide. Moreover, expression of the phosphorylation mutant (SPF1S289A) failed to result in stimulation of cholesterol synthesis in hepatoma cells, indicating that this serine residue (S289) has to be phosphorylated to stimulate cholesterol synthesis in whole cells.
Since GOLD domain stands for Golgi Dynamics domain, and the GOLD domain is thought to be involved in protein-protein interactions and intramembrane events, it was suggested that SPF associates with Golgi. One of the means to test this hypothesis was the construction of a GOLD domain deletion mutant (SPF1del111/SPF292). This truncated protein was expressed in rat hepatoma cells and indeed, was unable to stimulate cholesterol synthesis in these cells. This result revealed that the GOLD domain is necessary for the SPF stimulatory effect and suggested that interaction with Golgi may be required for SPF function. To determine if interaction with Golgi is required for stimulation of cholesterol synthesis by SPF1, studies with brefeldin A, which disrupts Golgi structures, were carried out. The stimulatory effect on cholesterol synthesis in SPF1-overexpressing cells was totally lost in brefeldin A-treated cells, which could be reversed by removal of the disaggregator, allowing reassembly of the Golgi bodies. These results indicated that SPF-mediated stimulation of cholesterol synthesis depends on intact Golgi and phosphorylation of SPF.

**Experimental Rationale**

SPF proteins belong to a family of lipid binding proteins and are thought to be involved in intracellular transport of small lipid molecules. The structure contains lipid binding domain and GOLD domain that is thought to be involved in bringing cargo from ER towards Golgi or in other protein-protein interactions. Previous studies have shown that the SPF1 protein is activated by phosphorylation and that Ser289 is required for this phosphorylation. Also, stimulatory the effect of SPF on cholesterol synthesis requires intact Golgi and the GOLD domain is thought to mediate this putative interaction.

SPF is presumably a transporter protein and therefore I hypothesized that in response to ligand binding or/and other stimuli it would redistribute within the cell. Two SPF mutants, one having serine 289 replaced by alanine, and the other missing the GOLD domain, are inactive, and therefore I hypothesized that, in contrast to the wild type
protein, these mutant SPF proteins would fail to localize correctly in the cell during cholesterol synthesis or upon phosphorylation.

To test this hypothesis, immunolocalization studies on hepatoma cells transfected with an SPF expression plasmid or SPF mutants. Transfected cells were treated with inhibitory and activating stimuli which were shown earlier to affect SPF activity (dbcAMP, brefeldin A, PKAi, Vitamin E). Since these treatments and alterations have a significant effect on the ability of SPF to stimulate cholesterol synthesis, I hypothesized that they will also engender changes in SPF localization inside the cell. Identification of the subcellular compartments to which SPF localizes in response to these treatments would provide insight on the mechanism of SPF function.
MATERIALS AND METHODS

Site directed mutagenesis by overlap-extension PCR

The PCR fragment (A-Mut-B) containing the desired mutation was obtained in a sequence of reactions depicted on the scheme in Fig. 4.1 and was used in the whole plasmid amplification reaction (WPA). A 50 μl WPA reaction consisted of: 50 ng of template DNA (purified plasmid containing gene to be mutated, in this case pc3.1SPF1), a primer pair (1 μl of A-Mut-B PCR fragment), 0.2 mM dNTPs, 1x Pfu buffer, and 2.5 U of Pfu Turbo DNA polymerase. Reaction conditions were: 95°C for 1 minute, followed by 18 cycles of: 95°C for 1 minute, 55°C for 1.5 minutes, and ending with 68°C for 9 minutes.

Cell culture

McARH7777 rat hepatoma cells were cultured in Dulbecco's Modified Eagle Medium (Gibco) supplemented with 1x Penicillin-Streptomycin mixture (Invitrogen) and 10% Fetal Bovine Serum (Invitrogen).

Plasmid transfections

The day before transfection, cells were plated on glass coverslips at a density of \( 5 \times 10^4 \) cells per cm\(^2\). On the second day plasmid DNA was transfected into the cells with use of Lipofectamine™ (Invitrogen) or Fugene6 (Roche Diagnostics). Lipofectamine transfections: Plasmid DNA was pre-complexed with Plus™ Reagent (Invitrogen) in serum-free medium at the ratio 1 μg:10 μl and incubated at room temp for 15 min. Medium-diluted Lipofectamine was added (2.5 μl per 1 μg of DNA) to form complexes with the mixture and incubated at room temperature for 15 min. The media was replaced with half of the recommended volume of serum and antibiotic-free DMEM
medium. The transfection mixture was added dropwise to the cells and they were allowed to incubate at 37°C. After 3 h the volume of medium was increased to normal volume with DMEM containing 20% FBS and 2% penicillin/streptomycin solution. **Fugene6 transfections:** Plasmid DNA was mixed with Fugene 6 reagent in serum-free medium at the ratio 1 µg:5 µl and complexes were allowed to form for 30 min at room temperature. The media was replaced with antibiotic-free medium containing 10% FBS and the transfection mixture was added dropwise to the cells.

**Immunofluorescence**

To determine the cellular localization of the expressed SPF protein, cells were stained with primary antibody against the hemagglutinin epitope (rabbit polyclonal HA-probe Y11) followed by a secondary antibody conjugated to the fluorophore, which, upon excitation, emits fluorescence with a maximum at the specified wavelength. For colocalization studies antibodies for organelle-specific proteins were used. Cells, once immunostained and mounted on the glass slides, were analyzed by confocal microscopy. Efficiency of transfection was also confirmed through X-gal staining on cells transfected with plasmid containing the lacZ gene.

**Isolation of primary hepatocytes**

Hepatocytes were prepared from mice as follows: mice were anesthetized with Ketamine and the liver was perfused via the portal vein with 30 ml of liver perfusion solution (Ca²⁺ and Mg²⁺-free HBBS, 10 mM HEPES, pH 7.4, 0.1 mg/ml gentamycin sulfate, and 0.5 mM EGTA) followed by 30 ml of collagenase solution (Ca²⁺ Mg²⁺-free HBBS, 0.06% collagenase, supplemented immediately before use with CaCl₂, to final concentration of 0.5 mM) at 3ml/min. The liver was removed, transferred into a tissue culture plate with collagenase solution and the liver capsule was removed, the
hepatocytes were dissociated by shaking and filtered through a double layer of gauze. The cells were pelleted at low speed in a microcentrifuge, washed twice, counted, and viability determined by trypan blue exclusion. Hepatocytes were plated on coverslips coated with collagen in 35 mm dishes at 0.5 \times 10^6 cells per 1 ml in DMEM containing 5% FBS. After 3 hr, the cells were washed and the medium was replaced with fresh medium. On the following day the hepatocytes were stained for immunofluorescent analysis.
RESULTS AND DISCUSSION

Site-directed mutagenesis of SPF

The coding sequence for the hemagglutinin (HA) epitope (YPYDVPDYA) was inserted at the carboxy termini of the SPF cDNAs previously cloned into the pc3.1DNA(-) plasmid (SPF1, SPF1 mutants and SPF3). Insertion of the HA tags was carried out with site-directed mutagenesis by overlap extension PCR, using the strategy depicted on the scheme below (Fig. 4.1). In brief, two fragments of a target sequence are amplified in two separate PCR reactions (A and B) by using, for each reaction, one universal (A or B) and one mutagenic primer, which contains desired insertion, deletion or point mutation (A-Mut, B-Mut). The two PCR products of these reactions were complementary at one end and they served as a new, overlapping template for the next reaction. DNA amplification on the overlapping template was performed with only the universal primers (A and B). The overlapped PCR product containing the desired mutation (A-Mut-B) was used as a primer pair for whole plasmid amplification of the original template. The product of this amplification contains mutated DNA; to eliminate the unmutated, original DNA template, digestion with the restriction enzyme DpnI was performed, taking advantage of its ability to cleave only methylated DNA. Unmethylated, mutated plasmid was used to transform competent cells. These cells were then grown on selective media, DNA isolated and sent for sequencing to identify positive clones. As a result, the following constructs were obtained: pc3.1SPF1-HA, pc3.1SPF1S289A-HA, pc3.1SPF1del111-HA, and pc3.1SPF3-HA.
Rat hepatoma cells (McARH7777) were grown in 6-well plates and on glass coverslips and pc3.1SPF1-HA cDNA was transfected into the cells with use Fugene 6 Reagent (Roche). On the second day post-transfection, the expression of SPF protein was confirmed by immunoblotting using an antibody to the HA epitope, as follows. The cells
grown on coverslips were fixed with ice-cold methanol and processed for immunofluorescence. Coverslips were incubated with primary antibody against the HA tag or against a marker of interest. To detect the signal, a secondary antibody conjugated to fluorochrome was used. The coverslips were mounted on glass slides and analyzed by fluorescent or confocal microscopy. Expressed SPF appeared to localize to the cytosol (Fig. 4.2).

Figure 4.2: SPF1-HA protein expressed in McARH7777 cells. A) HA probe (red); B) Grp94 marker for endoplasmic reticulum (green); C) DAPI nuclear stain (blue); D) merge of A, B and C.

To determine whether the heterologously expressed SPF protein changes its intracellular location in response to phosphorylation, transfected cells were treated with dbcAMP, a PKA stimulator, or 14,22-amide, a PKA inhibitor. No change in localization was observed upon either of these treatments (Fig. 4.3 E-H).
Since disruption of Golgi resulted in loss of the SPF-mediated stimulatory effect on cholesterol synthesis 22, SPF-transfected cells were treated with brefeldin A for 3 hours prior to immunostaining. Disruption of Golgi was confirmed by immunostaining for the Golgi marker GM130 protein (Fig. 4.3 J). No change in SPF subcellular localization was observed upon disruption of Golgi (Fig 4.3 I-L). Upon treatment with α-tocopherol SPF protein was reported to translocate from cytosol into the nucleus in Cos-7 cells 34. In transfected rat hepatoma cells SPF protein appeared to remain in cytosol upon addition of α-tocopherol (data not shown).
Figure 4.3: SPF1-HA protein expressed in McARH7777 cells, treated with dbcAMP and brefeldin A. Green - HA probe; red - Golgi marker (GM130); blue - DAPI nuclear stain.

Immunolocalization studies with mutant SPF proteins, previously shown to lose the ability to stimulate cholesterol synthesis, were then performed. Phosphorylation mutant (SPF1S289A) and GOLD domain deletion mutant (SPF1del111) were expressed in rat hepatoma and cells processed for immunofluorescence. Compared with wild-type
protein, no difference was observed in the subcellular localization of SPF1S289A mutant, as it remained in the cytosol (Fig 4.4 E-H). Interestingly, the Golgi domain deletion mutant, SPF1del111, localized to large vesicular formations around the nucleus, but which appear to be different from Golgi (Fig 4.4 I-L). A similar result was found for the protein-tyrosine phosphatase PTP-MEG2, in which a deletion mutant consisting of the Sec14 domain of this protein also associated with large vesicular structures surrounding the nucleus 15. This raises the possibility that the GOLD domain is not the only targeting signal on SPF, and that the Sec14 domain, assumed to be involved only in lipid binding, also contributes to the subcellular localization of these proteins. In support of this hypothesis, p45, the rat ortholog of SPF2, associates with large cytoplasmic vesicles, and this association is not disrupted by removal of the GOLD domain 48. Together these findings suggest that SPF targeting in the cell is a complex function of multiple components of this multidomain protein.
As mentioned earlier and shown in Fig. 4.3, heterologously expressed SPF protein localized to the cytosol and did not appear to move in response to any of tested treatments. Because expression from the transfected plasmid yields higher than normal levels of the protein in the cell, subcellular movement in response to various stimuli may not be evident, as the abundance of the protein may obscure the relocation of a small but important fraction of the protein. It is also possible that the very high levels of
expressed SPF protein overwhelm the cellular processing machinery, such as the phosphorylation pathway, resulting in a majority of protein that is not properly modified for activity. To address this possibility, I attempted to see where the endogenous SPF localizes. Unfortunately, McARH7777 cells express negligible amounts of SPF, which is undetectable by immunofluorescence with antibodies to SPF. Therefore, to assess localization of endogenous SPF the studies were carried out with mouse primary hepatocytes.

**Antibody specific for SPF protein**

Our custom-made chicken antibody against SPF was successfully used for immunoblotting, however the results from immunofluorescence studies were unsatisfactory. Various methods of fixation (4% paraformaldehyde, ice-cold methanol) and blocking (BSA, goat serum) were tested, as well as range of antibody concentrations. Despite these efforts, the background signal remained very high. The most likely reason for this appeared to be the purity of the antibody. Therefore, the antibody was purified by affinity chromatography.

Affinity chromatography takes advantage of specific binding between interacting molecules. The system for chromatographic purification is composed of a column with an immobilized ligand through which the mixture to be purified is passed. Only molecules having specific binding affinity to the ligand stay on the column and are later eluted. The appropriate ligand for antibody purification in this instance is SPF, and so large quantities of recombinant SPF1 are needed. As the previously purified protein exhibited some contamination when evaluated by SDS-polyacrylamide gel electrophoresis, the protein was further purified by gel filtration. Gel filtration, also called size-exclusion chromatography, separates molecules based on size. Multiple fractions were collected, analyzed by electrophoresis, and those containing only the 46-kD protein were pooled, resulting in significantly improved purity of the SPF1
preparation. For affinity chromatography the recombinant SPF protein was immobilized to a SulfoLink (SulfoLink, Pierce) column, taking advantage of free sulfhydryl bonds (cysteines) on SPF. The chicken SPF primary antibody was applied to the column, washed with several volumes of buffer, and eluted with a low pH glycine buffer and subsequently neutralized by addition of 1M Tris-HCl, pH 8.5, before use.

Affinity-purified chicken SPF antibody was tested by immunostaining of SPF-transfected cells. There was a marked increase in signal from transfected cells compared with signal obtained with non-purified antibody, however the background remained high in cells that did not express the transfected protein, and therefore unsatisfactory (Fig. 4.5).

Figure 4.5: Affinity-purified chicken SPF antibody shows significant background in immunofluorescent staining. Hepatoma cells transfected with SPF1-HA protein and stained with: A, affinity-purified chicken SPF antibody; B, HA probe; C, DAPI nuclear stain; and D, merge of A-C.
Subsequently new commercial antibodies to SPF became available. I tested goat polyclonal SPFα/β(N-18) and SPFα/β(N-14) antibodies (Santa Cruz Biotechnology, Inc.); however, they showed a very weak and not very specific signal on immunoblots. Fortunately, rabbit anti-human Sec14l2 antibody (GenWay Biotech, Inc.) yielded a strong and very specific signal on immunoblots as well as in immunofluorescent staining.

Before isolation of primary hepatocytes the expression of SPF in mouse and rat liver was confirmed by immunoblotting. A reasonable amount of protein was detected in both mouse and rat liver cytosol. This also demonstrated that the GenWay Sec14l2 antibody against human SPF detects proteins of the same size from mouse and rat (Fig. 4.6).

![Image of immunoblot](image_url)

**Figure 4.6:** SPF protein is present in rat and mouse liver cytosol. Immunoblot developed with rabbit anti-human Sec14l2 antibody (GenWay Biotech, Inc.).

Mouse primary hepatocytes were prepared as described in the Materials and Methods section and plated on collagen-coated coverslips. The next day the cells were fixed and processed for immunofluorescent staining (Fig. 4.7).

It should be noted that there was no negative control in studies on primary hepatocytes, as all were expected to express SPF. However, the conditions for immunofluorescence were optimized earlier on McARH7777 cells, where endogenous SPF protein could not be detected.
Figure 4.7: Endogenous mouse SPF protein (red) localizes to the cytosol. Red, Sec14l2 antibody signal; blue, DAPI nuclear stain.

It appeared that endogenous SPF protein, as anticipated, is present ubiquitously in the cytosol. To see if endogenous SPF protein moves in response to the treatments described earlier, cells were treated for 3 hours with dbcAMP, PKAi (inhibitor), brefeldin A, or α-tocopherol and processed for immunostaining.
**Figure 4.8:** SPF protein localizes to cytosol in mouse primary hepatocytes and does not relocate in response to various treatments. Treatments: A) none; B) 0.2 mM dbcAMP; C) 0.1 µM PKAi; D) 100 nM brefeldin A; E) 50 µM α-tocopherol. Green –Sec14l2 antibody signal; blue –DAPI nuclear stain.

Analysis by confocal microscopy showed no evident change in protein subcellular localization (Fig. 4.8).
Summarizing, I conclude that SPF either does not translocate in response to these treatments or that perhaps only a small portion of the protein was responsible for the effect on cholesterol synthesis and only this protein relocates within the cell, and that this limited movement cannot be detected by immunostaining. Perhaps subcellular fractionation and subsequent analysis of SPF content after these treatments would reveal relative changes in localization, as well as the membrane structures involved.

This finding was a bit surprising, as the effect of these treatments clearly affected the ability of SPF to stimulate cholesterol synthesis \textsuperscript{21, 22}. It would appear that SPF is relatively highly expressed in hepatocytes, an observation consistent with early enzymology studies that demonstrated an abundance of this protein in cytosol. The abundance of this protein would obscure subcellular changes in a small proportion of the protein.
Chapter 5: REGULATION OF SPF EXPRESSION IN RAT HEPATOMA CELLS

INTRODUCTION

Shibata et al. 24 found that plasma lipids levels were unchanged in SPF-null mice, despite elevated levels of HMG-CoA synthase and squalene monooxygenase in liver. However, during fasting plasma cholesterol levels decreased significantly in SPF-null mice, but not in wild-type mice. Fasting decreased hepatic cholesterol synthesis in both wild-type and SPF-null animals, with a greater reduction in SPF-null mice. The decrease in cholesterol synthesis likely resulted from a pronounced decrease in the expression of the cholesterologenic enzymes HMG-CoA synthase, HMG-CoA reductase, and squalene monooxygenase in both wild-type and knockout mice. Notably, in fasted wild-type mice, expression of SPF mRNA and protein was elevated.

The data from SPF-null mice strongly suggests that SPF is upregulated upon fasting to compensate, at least partially, for the decrease in cholesterol synthesis. The reason for this compensatory increase is not clear. In agreement with these observations, previous data from our lab have shown that SPF activity was decreased by about 50% in rats fed high fat diet and returned to normal upon return to normal chow diet 21. The decrease in expression was suggested to explain the decrease in SPF activity in liver preparations from the high-fat diet animals, since activity could not be increased by in vitro phosphorylation 21, which this laboratory has shown to be able to activate SPF 21, 22. These results are in agreement with studies from SPF-null mice and suggest that upregulation of SPF expression during fasting might be attributed, at least in part, to removal of lipids from the diet.
The gene that was deleted in SPF-null mice is a mouse SPF (mSec14l2), an ortholog of the human SPF1 gene (hSec14l2) with 93% identity. The studies described below were conducted in rat hepatoma cells, in which there are two established rat orthologs of SPF: rat SPF (rSec14l2, with 94% identity to human SPF1) and rat SPF-like protein (rSec14l3/p45, with 97% identity to human SPF2).

SREBPs, Sterol Regulatory Element Binding Proteins, are membrane-bound transcription factors that are master regulators of fatty acid and cholesterol biosynthesis. SREBP-1a regulates enzymes responsible for biosynthesis of fatty acids and cholesterol, SREBP-1c is specific for fatty acid biosynthesis, and SREBP-2 regulates cholesterol biosynthesis. While SREBPs activate the transcription of genes responsible for sterol and fatty acid synthesis in response to low levels of these lipids, the LXR-dependent regulatory pathway is activated in response to elevated cellular sterols. Oxysterols, which are oxidized derivatives of cholesterol, are the known endogenous ligands for the LXR nuclear receptor. The LXRα receptor increases the expression of cholesterol efflux genes and decreases cholesterol uptake and synthesis by suppressing SREBP activation, and accelerating HMG-CoA reductase degradation.49

The following studies were undertaken to gain insight into the mechanisms that govern the expression of SPF proteins.

**Experimental Rationale**

Fasting decreased hepatic cholesterol synthesis in both wild-type and SPF-deficient animals with a greater reduction in knockout mice.24 The decrease in the cholesterol synthesis rate was accompanied by a pronounced decrease in the expression of cholesterolgenic enzymes: HMG-CoA synthase, HMG-CoA reductase, and squalene monooxygenase. Expression of the SPF transcript and protein was elevated in wild-type mice during starvation. I hypothesized that serum deprivation similarly will induce
expression of SPF in a hepatoma cell line, mimicking starvation in mice, facilitating studies on the regulation of SPF in a cell culture system. Putative nuclear receptors regulating SPF expression also will be identified.
MATERIALS AND METHODS

Materials

Cholesterol, hydroxypropyl-β-cyclodextrin, 25-hydroxycholesterol, 22(S)-hydroxycholesterol, and GW3965 were purchased from Sigma-Aldrich, Inc.

Preparation of cholesterol: cyclodextrin complexes. Cholesterol was dissolved in ethanol (5 mg/ml) and 1 ml of that solution was dried under liquid nitrogen. Two ml of 5% hydroxypropyl-β-cyclodextrin solution in water was added to the cholesterol and the mixture was sonicated in a water bath at room temperature until the solution was clarified.

Cell culture

McARH7777 rat hepatoma cells were cultured in Dulbecco's Modified Eagle Medium (Gibco) supplemented with 1x Penicillin-Streptomycin mixture (Invitrogen) and 10% Fetal Bovine Serum (Invitrogen), unless otherwise stated.

Preparation of Lipid-Deficient Serum. One volume of Fetal Bovine Serum (Invitrogen) was mixed with one volume of diethyl ether and vortexed vigorously. The ether fraction containing the lipids was removed and the delipidated serum was used for cell culture.

RT-PCR

Total RNA was isolated from cells with use of TRIzol reagent (Invitrogen) and the concentration of RNA was measured spectrophotometrically with use of the Nanodrop instrument (Thermo Scientific). The reverse transcriptase reaction and real-time PCR were performed as described in Materials and Methods in Chapter 2. Gene-specific primers for real-time PCR were designed using ABI Prism Primer Express software.
(Applied Biosystems). The sequences of the primers are displayed in Table 5.1. Results were analyzed with use of Comparative Ct method.

Table 5.1: Gene-specific primers used in real-time PCR.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
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<tbody>
<tr>
<td>B actin</td>
<td>Fw: 5’-CCGTGAAAAGATGACCCAGATC-3’</td>
</tr>
<tr>
<td></td>
<td>Rv: 5’-AATGCCAGTGGTACGACCAGA-3’</td>
</tr>
<tr>
<td>HMGcr</td>
<td>Fw: 5’- TCATCCTCACGATAACCCGGT-3’</td>
</tr>
<tr>
<td></td>
<td>Rv: 5’- GGCCGGCAATACCCAAAAT-3’</td>
</tr>
<tr>
<td>rSec14l2</td>
<td>Fw: 5’- AATTCCCACATGGTCCCTGAG-3’</td>
</tr>
<tr>
<td></td>
<td>Rv: 5’- ACTTTCTTGGCATGGATGAAGC-3’</td>
</tr>
<tr>
<td>rSec14l3</td>
<td>Fw: 5’- CAGTATGAGCCTCGGTGCAG-3’</td>
</tr>
<tr>
<td></td>
<td>Rv: 5’- GCTCCATCAGATGAGAACTGCC-3’</td>
</tr>
</tbody>
</table>
RESULTS AND DISCUSSION

As fasting up-regulated the hepatic expression of SPF in mice, I wanted to generate an in vitro model using cultured hepatoma cells. To do this I started by changing the concentration of glucose in the media and looking at its effect on SPF and HMG-CoA reductase expression in treated cells. McARH7777 rat hepatoma cells are usually cultured in high glucose (4.5 g/L) media; I cultured these cells at a low concentration of glucose (1 g/L). After 48 hours RNA was isolated from the cells and the expression of HMG-CoA reductase and the two rat SPF genes (Sec14l2 and Sec14l3) was assessed by real-time PCR. Although I anticipated an increase in SPF expression when the glucose concentration was reduced (as is seen in fasting), lowering the glucose concentration in the media had no significant effect on mRNA levels for HMG-CoA reductase or either SPF gene; indeed, a trend toward a decrease in expression was seen (Fig. 5.1, orange bars). Since this data showed that a reduction in the glucose concentration does not result in the upregulation of SPF genes, I concluded that these conditions may not appropriately mimic the fasting state in mice. This led to the conclusion that fasting may alter other components of serum, including hormones and other nutrients; changes in the level of these components may be responsible for the changes seen in SPF expression in fasted animals. Since very little is known about SPF regulation, and serum has multiple components, I decided to explore the role of serum in SPF expression.

When cultured hepatoma cells were maintained in serum-free medium for 48 h the expression of both rat SPF genes was increased while HMG-CoA reductase expression remained unchanged (Fig. 5.1, red bars). The increase in SPF transcripts was less than 100%, but statistically significant. Interestingly, when cells were cultured in low glucose and serum-free media the increase in both SPF transcripts was more dramatic (Fig. 5.1, brown bars), indicating that removal of glucose potentiates the effect serum.
This result supported the hypothesis that the increases in SPF expression induced by fasting might be mediated by changes in serum components.

Figure 5.1: Serum removal results in an increase in the expression of SPF proteins in rat hepatoma cells; this effect is potentiated by removal of glucose. Cells were cultured for 48 h in high or low glucose, with or without FBS. HG, high glucose; LG, low glucose; FBS, fetal bovine serum; HMGcr, HMG-CoA reductase. Statistical analysis performed by ANOVA, (*) p<0.05; (**) p<0.0001; N, number of experiments run in duplicate.

To address this possibility, I have tried to identify the components of serum that could alter SPF gene expression. Since SPF is known to upregulate cholesterol synthesis, I hypothesized that a decrease in serum cholesterol levels might upregulate SPF expression. As expected, when hepatoma cells were cultured in medium containing serum from which the majority of lipids had been removed (lipid-deficient serum, LDS), the expression of both SPF genes and HMG-CoA reductase was increased, although the increase was not statistically significant (Fig. 5.2).
By the same logic, since high cholesterol levels inhibit cholesterol synthesis through negative feedback regulation, it was anticipated that SPF and HMG-CoA reductase expression would be suppressed upon supplementation of the media with a mixture of cholesterol:25-hydroxycholesterol (10:1 μg/ml). Both cholesterol and 25-hydroxycholesterol downregulate cholesterol biosynthesis via inhibition of SREBP processing 51. Indeed, as shown in Fig. 5.3, supplementation of the media with a mixture of cholesterol:25-hydroxycholesterol resulted in a decrease in the expression of HMG-CoA reductase and both SPF genes by up to 50%. These results indicate that the expression of both SPF genes depends on cholesterol availability, and suggest that the upregulation of SPF gene expression upon serum deprivation may result, at least in part, from the loss of cholesterol in the medium.
As shown above, a mixture of cholesterol:25-hydroxycholesterol decreased the expression of SPF genes in the presence and absence of serum. Since both of these compounds inhibit the SREBP pathway but 25-hydroxycholesterol is also a ligand for LXR receptor\textsuperscript{52}, these results raised the question as to whether SPF gene expression is regulated by SREBP or LXR. To test the role of the LXR in SPF regulation, a specific LXR agonist, GW3965, was added to cells cultured in the absence of serum. If LXR activation suppresses SPF expression the addition of an LXR agonist should prevent or reverse the induction of SPF gene expression by serum deprivation. However, as shown in Fig. 5.4, the LXR agonist was unable to prevent the increase in SPF expression in these cells. This
result suggests that cholesterol (and 25-hydroxycholesterol) act through the SREBP pathway, rather than the LXR pathway, to suppress SPF expression.

Figure 5.4: LXR ligand does not change the effect of serum and glucose removal on SPF expression. Cells were cultured in low-glucose media. FBS-fetal bovine serum; GW3965, LXR receptor agonist; n = 2.

Summarizing, my studies in rat hepatoma cells have shown that expression of both rat SPF genes is upregulated in response to serum removal, which is potentiated by removal of glucose. Also, my studies have shown that lipids, most likely cholesterol, are the likely components of serum whose absence contributes to the increase in SPF transcripts. These results are consistent with a role for SPF in upregulating cholesterol synthesis in hepatoma cells.

To further explore the effect of serum and glucose removal on SPF expression, I attempted to identify transcription factors that regulate the transcription of the SPF
genes. LXR, SREBP and/or PPARα were considered to be likely candidates. My studies show that LXR is most likely not the transcription factor governing expression of SPF genes in my cell culture system, as addition of an LXR agonist did not suppress SPF expression. The possibility of regulation via SREBP remains to be tested. Moreover, the possibility that SPF gene expression is regulated differently in cell culture than in a whole animal must also be considered.
Chapter 6: SUMMARY OF RESEARCH

SPF is a cytosolic protein known to stimulate cholesterol biosynthesis. HMG-CoA reductase, squalene monooxygenase and oxidosqualene cyclase are the three enzymes of the cholesterol biosynthetic pathway that are known to be stimulated by SPF. The mechanism of SPF action is not yet well understood. More recent studies have revealed that there are three highly similar SPF genes in the human genome. They encode three homologous proteins: SPF1, SPF2 and SPF3. The protein that has been studied the most to date is SPF1. Little is known about the expression of the other two SPF proteins and nothing is known about their function. To gain insight into possible functions for these proteins, I utilized three different approaches to determine which tissues express SPF genes. In all, over 75 tissues were screened for expression of SPF mRNA, using highly sensitive probes designed to detect each SPF gene transcript with high specificity. SPF expression was most evident in liver, stomach, and mammary tissue, with levels in liver being at least 50-fold higher than those found in other tissues. My studies further show that, of the three SPF transcripts, SPF1 is the most abundant, followed by approximately 20-fold lower expression of SPF3 and negligible expression of SPF2. Although I had hoped to uncover differential expression of these genes in various tissues suggestive of functional differences, the expression patterns did not support this hypothesis. In all tissues where SPF was present, SPF1 expression dominated, suggesting that SPF2 and SPF3 were either non-functional, of marginal importance, or were expressed only in specific conditions or developmental stages. Further studies will be needed to identify any such conditions.

The SPF2 transcript could only be detected in human liver and its level was very low when compared with SPF1 and SPF3. Full-length SPF2 could not be cloned and
therefore I was unable to assess its ability to stimulate cholesterol synthesis. The fact that p45 protein, a rat ortholog of human SPF2, was able to stimulate cholesterol synthesis \(^{38}\), suggests that human SPF2 might have similar ability. This question remains to be answered in future studies. However, given the negligible expression levels in all tested tissues, it is reasonable to discount SPF2 as a factor contributing to the stimulatory effect of SPF on cholesterol biosynthesis in liver. The possibility remains that SPF2 is expressed in other, as yet untested tissues, or that its expression is induced in response to certain stimuli or conditions. Both of these possibilities remain to be tested. My studies revealed that human SPF3 protein is capable of stimulating cholesterol synthesis in cell culture, although it appears somewhat less effective than SPF1, and is unable to stimulate squalene monooxygenase activity in microsomal preparations. This finding suggests that SPF3 may act only on HMG-CoA reductase, in contrast to SPF1, which stimulates both squalene monooxygenase and HMG-CoA reductase, as well as oxidosqualene cyclase. The possibility that SPF3 activates HMG-CoA reductase \textit{in vivo} remains to be determined. Overall, the limited expression of SPF3 in all tissues including liver suggests that, like SPF2, the protein does not play a significant role in regulating cholesterol synthesis, although it remains possible that its expression is upregulated, perhaps in select tissues, in response to environmental, hormonal, or developmental stimuli. In the absence of such data, I conclude that SPF1 should be considered as a main human SPF homolog responsible for stimulating cholesterol synthesis in liver.

SPF was shown to require phosphorylation and the presence of Golgi structures in order to stimulate cholesterol synthesis in rat hepatoma cells \(^{22}\). It was hypothesized that, once activated by phosphorylation, SPF associates with some membranous compartment, perhaps the endoplasmic reticulum (ER), in order to stimulate the enzymes of the cholesterologenic pathway, which are membrane-bound proteins. I anticipated that in response to protein kinase A-mediated phosphorylation induced by adding dbcAMP to rat hepatoma cells, SPF will associate with some membrane
compartment and that this change in localization would be captured by my immunocytochemistry studies; by comparison, the phosphorylation mutant SPF1S289A, which cannot be phosphorylated, and the wild-type protein in cells treated with a PKA inhibitor, will fail to translocate. However, my studies in rat hepatoma cells revealed no noticeable change in protein localization inside the cell in response to these treatments.

Disruption of Golgi by brefeldin A in rat hepatoma cells was shown to abolish the stimulatory effect of heterologously expressed SPF protein on cholesterol synthesis. Similarly, SPF protein lacking the GOLD domain (SPF1del111), thought to mediate Golgi interactions, was also unable to stimulate cholesterol synthesis. I anticipated that these effects would be reflected by changes in protein cellular localization. Treatment with brefeldin A did result in disruption of Golgi structures, but no change in SPF protein localization was observed. Transfections with a GOLD domain mutant resulted in a protein localizing mainly to the large vesicular structures around nuclei, different from Golgi, suggesting that remaining Sec14 domain may contain signal targeting to these vesicular structures.

To rule out the possibility that the change in subcellular localization could not be detected due to the abundance of overexpressed protein in transfected cells, or because the recombinant protein behaves differently than the endogenous one, I decided to test the effect of the above treatments on the endogenous SPF. To do so I isolated primary hepatocytes from mice and performed these treatments on these cells; once again, however, I did not observe a change in the SPF subcellular location. These results led to the conclusion that SPF either does not translocate in response to these treatments or that perhaps only a small portion of the protein was responsible for the effect on cholesterol synthesis and only this protein relocates within the cell, and that is why the movement cannot be detected by immunostaining. Perhaps subcellular fractionation and subsequent analysis of SPF content after these treatments would reveal relative changes in localization, as well as the membrane structures involved.
SPF-null mice had previously been shown to develop normally, and their plasma lipids are unchanged. However when tested by food restriction, plasma cholesterol levels decreased significantly in these mice, but not in wild-type mice. Interestingly, in starved wild-type mice expression of mouse SPF protein was elevated. These findings strongly suggested that SPF is a “buffering” protein, responsible for maintaining plasma cholesterol levels during short-term starvation. In my studies in rat hepatoma cells I found that expression of both rat SPF genes is upregulated in response to serum removal, which is potentiated by removal of glucose. This effect of glucose is puzzling; glucose removal potentiated the effect of serum removal on SPF gene expression, but glucose removal alone did not significantly increase SPF expression. My studies have shown that lipids/cholesterol are the likely components of serum whose absence contributes to the increase in SPF transcripts; first, when hepatoma cells were cultured in delipidated serum SPF transcripts were increased; and second, supplementation of media with a mixture of cholesterol:25-hydroxycholesterol decreased the levels of SPF and HMG-CoA reductase transcripts. These results are consistent with a role for SPF in upregulating cholesterol synthesis in hepatoma cells. I also investigated the possible role of insulin in SPF regulation, but media supplementation with insulin did not appear to alter SPF gene expression.

To further explore the effect of serum and glucose removal on SPF expression, I attempted to identify transcription factors that regulate the transcription of SPF genes. LXR, SREBP and/or PPARα were considered to be likely candidates. Peroxisome proliferator-activated receptor alpha (PPARα) was recognized as critical regulatory element directing cellular responses to fasting. In fact, Shibata et al. showed that during starvation SPF expression is elevated in wild-type mice, but not in PPARα-null mice, suggesting that PPARα acts upstream of SPF expression. I have begun to test this hypothesis in our cell culture model by treating cells with PPARα agonists, which were expected to induce SPF gene expression. Preliminary data (not shown) did not support this hypothesis, but further investigation is needed to rule out this hypothesis.
completely. The possibility that SPF gene expression is regulated differently in cell culture than in a whole animal must also be considered. My studies show that LXR is most likely not the transcription factor governing expression of SPF genes in my cell culture system, as addition of an LXR agonist did not suppress SPF expression. The possibility of regulation via SREBP remains to be tested.

The mechanism of the SPF stimulatory effect on cholesterol synthesis remains to be elucidated. The fact that this protein stimulates three different enzymes of the cholesterolgenic pathway implies some general mechanism. All three enzymes are membrane-bound proteins and it was demonstrated earlier that, at least for squalene monooxygenase, SPF does not stimulate the solubilized enzyme, but only the membrane-bound form, suggesting some membrane-dependent event. Notably, SPF has two relevant domains: Sec14, a lipid binding domain, and a GOLD domain, proposed to mediate protein-protein interactions involved in Golgi function. It is predicted that such two-domain proteins may serve as adaptor proteins in assembling protein complexes on membranes, or perhaps help in cargo packaging into membrane vesicles. In fact, p45 protein, the rat homolog of human SPF2, was shown to be concentrated in secretory vesicles in COS-1 cells and secreted from the cell. In further support of this contention is the observation that the most likely ligand for SPF, phosphatidylinositol (PI), is a lipid that resides in the membrane, and therefore to bind this ligand SPF has to associate with phosphatidylinositol-rich membranes. Since PI was identified as the most likely ligand for SPF, and there are several phosphorylated derivatives of PI in the cell, it should be further explored whether one of these phosphorylated PIs has a higher affinity for SPF than PI. In support of this hypothesis lies the finding that PI(3,4,5)P₃ was identified as a lipid ligand of rat p45 protein. Identification of a true physiologic ligand would be helpful in further investigations on the mechanism of action of SPF.

Also, the link of SPF protein with phosphatidylinositol-3-kinase (PI3K) remains to be explored. SPF was earlier coprecipitated with PI3K and was shown to decrease the
activity of this kinase \textsuperscript{31}. Later, another study showed that SPF inhibits prostate cancer cell growth via inhibition of PI3K/Akt pathway \textsuperscript{40}. It would be interesting to test whether overexpression of SPF in hepatocellular carcinoma cells would also result in inhibition of cell division. Indeed, SPF expression in rat hepatoma cells is very low when compared with normal liver tissue, which is consistent with this hypothesis. Presently it is unclear if the SPF inhibitory effect on the PI3K pathway is linked to its ability to stimulate cholesterol synthesis; further studies in this direction are warranted.

In the light of current knowledge on SPF, it is reasonable to think that SPF participates in the assembly or trafficking of some membrane vesicles, perhaps containing substrates for the enzymes it stimulates; or by influencing the properties of the ER membrane in such a way that it creates more favorable conditions for the catalyzed reactions to occur. It is also possible that the stimulatory effect on cholesterol synthesis is not the primary function of SPF, but rather a “collateral” effect of another action. Also, the stimulatory effect on cholesterol synthesis was shown so far only in liver-derived cells, making it possible that it is the only site of this type of SPF activity. Therefore, given the several different reports on SPF function, including its ability to inhibit the PI3K pathway \textsuperscript{31,40}, its ability to retain vitamin E inside the cell \textsuperscript{40}, and its ability to stimulate enzymes of cholesterol biosynthetic pathway, it would be reasonable to think that perhaps the function of SPF is cell-type specific, and depends on the availability of other proteins with which SPF interacts.
REFERENCES


17. National Center for Biotechnology Information (NCBI online database).


46. Mokashi, V., Supernatant Protein Factor: insights into its regulation and ability to stimulate cholesterol synthesis *in vitro* and in cell culture, in *Department of Toxicology*, University of Kentucky, 2004.


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