Identification of the RNA Cis-Elements that Interact with SRp30a to Regulate the Alternative Splicing of Caspase 9 Pre-mRNA

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Virginia Commonwealth University

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IDENTIFICATION OF THE RNA CIS-ELEMENTS THAT INTERACT WITH SRP30A TO REGULATE THE ALTERNATIVE SPLICING OF CASPASE 9 PRE-MRNA

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

by

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Bachelor of Science, Bucknell University, 1995
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August 2005
Acknowledgement

It has been an extraordinary privilege to work in the Chalfant laboratory this past year. Dr. Chalfant’s years of experience and keen intellect were critical in order for me to grasp the concepts of molecular biology related to my research. It was very clear to me from the first day that I met him that he was both jovial and passionate about his research, and he was pragmatic as well. His pragmatism and ability to make complicated concepts simple were invaluable to me in my research. His great sense of humor will also be missed.

Other members of the Chalfant lab were very helpful to me as well. I would like to acknowledge Autumn Massiello, Shanaka Wijesinghe, Chaminda Fernando, Nadia Lamour, and Preeti Subramanian for their generous assistance with experimental design and techniques. Special thanks goes to Chaminda Fernando for teaching me cell culture and transfection techniques and Autumn Massiello for teaching me EMSA and RT-PCR techniques.

Finally, I would like to thank all members of the faculty of the Biochemistry Department at VCU for their support during the last two years. Your encouragement and belief in me was critical to my successes during my stay here. I will always remember it and appreciate it.
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Abstract

IDENTIFICATION OF THE RNA CIS-ELEMENTS THAT INTERACT WITH SRP30A TO REGULATE THE ALTERNATIVE SPlicing OF CASPASE 9 PRE-MRNA

By Prabhat Mukerjee, M.S.

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

Virginia Commonwealth University, 2005

Major Director: Dr. Charles E. Chalfant, Ph.D.
Assistant Professor, Biochemistry

Studies have shown that the alternative splicing of caspase 9 and the phospho-status of SR proteins, a conserved family of splicing factors, are regulated by chemotherapy and de novo ceramide via the action of protein phosphatase-1 (PP1). Two RNA splice variants are derived from the caspase 9 gene, pro-apoptotic caspase 9a and anti-apoptotic caspase 9b, via alternative splicing by either the inclusion or exclusion of an exon 3, 4, 5, and 6 cassette. In this study, the link between SR proteins and the alternative splicing of caspase 9 was established. Sequence analysis of the exon 3, 4, 5, and 6 cassette of the caspase 9 gene identified five possible high affinity sequences for interaction with
the SR protein, SRp30a, a well-established regulator of exon inclusion/exclusion. Replacement mutagenesis identified purine-rich sequences between exons 4 and 5 and within exon 6 as important for binding SRp30a and required for expression of the caspase 9a splice variant. *In vitro* binding assays coupled with competitor studies demonstrated specific binding of RNA trans-acting proteins and SRp30a with these sequences. Furthermore, SDS-PAGE analysis of cross-linked RNA trans-acting factors with these possible RNA cis-elements revealed the specific binding of an approximate 66, 56, 45, and 38 kDa protein/protein complex to these sequences. A previous application of RNAi technology to downregulate SRp30a in A549 lung adenocarcinoma cells induced an approximately 75% decrease in SRp30a expression and induced a dramatic change in the ratio of caspase 9a/caspase 9b. Therefore, these studies have identified SRp30a as a major regulator of the alternative splicing of caspase 9 directly linking *de novo* ceramide generation, PP1, and SRp30a as the signal transduction pathway regulating the expression of caspase 9.
CHAPTER 1 Introduction

Overview of Apoptosis and the roles of Caspase 9a and Caspase 9b

Apoptosis (used here interchangeably with programmed cell death) is a mechanism (or group of mechanisms) by which cells execute endogenous programs of cell death, often in response to adverse external or internal signals or sources of injury. In the case of cancer, interference with this system of programmed cell death can lead to expansion of deleterious cells. Apoptosis is regulated by multiple factors through complex mechanisms. It has become well established that many inducers of apoptosis (e.g. chemotherapy) activate caspases, and that the activation of these cysteine proteases is perhaps the point of irreversible commitment to the onset of apoptosis. In normal, healthy cells, a protein known as cytochrome \( c \) is sequestered between the inner and outer membranes of mitochondria (1). In the presence of apoptotic death inducing stimuli, such as chemotherapeutics or oxygen free radicals, monomers of a protein known as Bax migrate from the cytosol to the outer membranes of the mitochondria, homodimerize, and form a channel permitting ions to flow into the intermembranous space (1). This ion flux, through an unknown mechanism, triggers cytochrome \( c \) to be released into the cytosol where it binds to an adaptor protein known as Apaf-1. Apaf-1 then binds pro-caspase 9 and using the energy from the hydrolysis of ATP, pro-caspase 9 autoproteolyzes into the active
caspase 9 (2). The trimeric complex of pro-caspase 9 with cytochrome c and Apaf-1, known as the apoptosome, is shown in Figure 1. Caspase 9 is a member of the caspase family of proteases that functions as an initiator caspase. Active caspase 9 cleaves pro-caspase 3 into the active caspase 3 which cleaves additional substrates and leads to cell death (2).

Caspase 9 has two antagonistic isoforms in programmed cell death: the pro-apoptotic caspase 9a and the pro-survival caspase 9b as shown in Figure 2. The caspase 9 gene is composed of eight introns and nine exons. Of these nine exons, the exon 3, 4, 5, and 6 cassette encodes for the catalytic domain of caspase 9. If this cassette is included, the caspase 9a splice variant is produced and if it is excluded, the caspase 9b splice variant is produced. The caspase 9b variant lacks the catalytic protease domain and acts as a dominant negative to the caspase 9a splice variant such that the translated caspase 9b competes for Apaf-1 binding and also heterodimerizes with caspase 9a preventing it from being activated. In this way, caspase 9b acts as an anti-apoptotic factor. This suggests that alternative splicing, such as the alternative exon inclusion of caspase 9, may be an important mechanism in the regulation of cell fate.
Figure 1. Overview of the role of Caspase 9 in apoptosis.
The activation of caspase 9 occurs by pro-caspase 9 forming a trimeric complex known as the apoptosome with two other proteins, apaf-1 and cytochrome c. This trimeric complex utilizes the energy released from the hydrolysis of ATP to cleave pro-caspase 9 into an activated caspase 9 and initiating the apoptotic cascade.
Figure 2. Overview of Caspase 9 structure and its splice variants. Two splice variants can be formed from caspase 9 pre-mRNA, caspase 9a and caspase 9b. Determination of which splice variant is produced depends on whether the exon 3, 4, 5, 6 cassette is included or excluded. When the exon 3, 4, 5, 6 cassette is included during the splicing process, the pro-apoptotic caspase 9a mRNA is expressed. When the exon 3, 4, 5, 6 cassette is excluded during the splicing process, the anti-apoptotic caspase 9b mRNA is expressed.
Role of Ceramide in Apoptosis

The bioactive sphingolipid, ceramide, is an important regulator of various stress responses, apoptosis, and cellular senescence, and the formation of ceramide from the hydrolysis of sphingomyelin or from de novo pathways has been observed in response to agonists such as tumor necrosis factor-α, γ-interferon, 25-dihydroxyvitamin D3, interleukin-1, ultraviolet light, heat, chemotherapeutic agents, fatty-acid synthase antigen, and nerve growth factor. When these agonists have induced the formation of ceramide, there are several targets of ceramide that are inducers of apoptosis as shown in figure 3. These targets include ceramide activated protein kinase (CAPK), cathepsin D, and serine/threonine protein phosphatases PP1 (protein phosphatase 1) and PP2A (protein phosphatase 2A) also known as ceramide activated protein phosphatases (CAPPs) (3).

Direct Targets of Ceramide

The prominent role of ceramide as a regulator of cellular mechanisms necessitated the identification of these direct target enzymes previously mentioned. Cathepsin D has been shown to play an important role in proteolysis as well as apoptosis. Heinrich et al. found that cathepsin D is a direct target for ceramide and interaction with ceramide was necessary to cleave pro-cathepsin D into active cathepsin D (3). Studies by Kagedal et al. have shown that active cathepsin D, in response to oxidative stress, translocates from the lysosome and that activation of caspase 3 is dependent on this activation and translocation (4). It has been found that PKC-zeta (protein kinase C zeta) interacts directly with
ceramide thereby modulating the response to TNF (15). The resulting activation of PKC-zeta leads to the enhancement of splicing by increasing phosphorylation of hnRNPA1 leading to nuclear translocation and increased RNA binding affinity (16, 17). Kolesnick et al. have reported on a ceramide-activated protein kinase (CAPK) in A-431 human epidermal carcinoma cells and HL-60 human promyelocytic leukemia cells, but the specificity of ceramide activation of CAPK, nor its role in intracellular signaling, have been defined (5). Ceramide-activated protein phosphatase (CAPP), belongs to the family of serine/threonine protein phosphatases (6). CAPP was activated by ceramides that carried different lengths of N-linked fatty acyl groups and was not activated by sphingomyelin, sphingosine, or dihydroceramide (6). Also, CAPP activation in vitro closely matches the specificity of various cellular activities of ceramide, including apoptosis (6). Ceramide-induced apoptosis is inhibited by okadaic acid, an inhibitor of serine/threonine protein phosphatases, which is additional evidence for a role of CAPP in mediating ceramide responsiveness (6). There are two types of ceramide-activated protein phosphatases, which include the serine/threonine-specific protein phosphatases PP1 and PP2A (7). Both PP2A and PP1 have been reported to be targets for natural ceramides in vitro and in vivo (7).
Figure 3. Overview of the role of ceramide in apoptosis. Several putative and direct targets of ceramide action have been identified. Of these, those that couple ceramide generation with downstream inducers of apoptosis include: ceramide activated protein kinase (CAPK), cathepsin D, and serine/threonine protein phosphatases PP1 (protein phosphatase 1), PP2A (protein phosphatase 2A), and PKC (protein kinase C) (3).
Ceramide Metabolism

Formation of ceramide from the agonist induced hydrolysis of sphingomyelin (8) was shown in HL-60 human promyelotic leukemia cells treated with ercalcitriol. This and other agonists that serve to induce the hydrolysis of sphingomyelin suggests that a common theme of this group of agonists is associated with their different effects on growth suppression (6). In different cell systems, the ceramide formed downstream from these agonists induces either cell differentiation, cell-cycle arrest, apoptosis or cell senescence, and this suggests a role for ceramide in growth arrest and suppression (6).

The de novo pathway of ceramide synthesis has emerged as being responsive to agonist stimulation (6) with ceramide generated by this pathway capable of exerting biological actions (6). As shown in figure 4, condensation of serine and palmitoyl CoA by serine palmitoyltransferase (SPT) initiates the pathway and generates ketosphinganine which is then reduced, N-acylated, and desaturated by addition of the 4–5 trans double bond in a series of enzymatic steps, leading to the generation of ceramide (6). Ceramide can also be synthesized by ceramide synthase acting on sphingosine, and the reverse step, the synthesis of sphingosine can be accomplished by ceramidases acting on ceramide. In addition ceramide can be synthesized from sphingomyelin using sphingomyelinase and the reverse can be accomplished using sphingomyelin synthase.
Figure 4. Overview of Ceramide Metabolism (3)
The Role Of SR Proteins In Splicing

SR proteins are a family of arginine/serine rich domain containing proteins with both roles in constitutive RNA splicing and as regulators of alternative pre-mRNA processing (9). Spliceosome assembly requires many non-snRNP protein factors including members of the SR protein family (10). Each SR protein contains one or two N-terminal RNP-type RNA binding domains (RBDs) and a C-terminal domain enriched in Arg-Ser dipeptide repeats (RS domain) (10), and SR proteins were shown to function in pre-mRNA splicing as both general and alternative splicing factors (10). In splicing deficient HeLa extracts, they were found to allow the splicing of several different pre-mRNAs (10), and in another case, SR proteins were shown to modulate alternative splicing by affecting the usage of competing 5' or 3' splice sites in a concentration dependent manner (10). SR proteins are also required for the activity of splicing enhancer elements, which are RNA sequences, most frequently found in exons that are needed to activate certain weak splice sites (10). Specifically, the SR proteins, SRp30a and SRp30b have been shown to interact with U1 snRNP-specific 70K protein (U1 70k), and the RS domains of both proteins are required for this interaction (10). SRp30a has also been shown to bind 5' splice sites (10). These protein-protein and protein-RNA interactions have the potential to bring the two splice sites together across either the intron or the exon (10) and as splicing activators, SR proteins bind specific sequences in RNA enhancers and form complexes with other proteins (6). SR proteins are phosphorylated extensively in vivo and phosphorylation takes place mainly on serine residues within the RS domain (10). Addition of the serine
threonine phosphatase, PP1, to nuclear extract inhibits spliceosome assembly and phosphorylation of SR proteins is required in the progression of A complex to B complex of the spliceosome (10). Furthermore, alternative 5' splice selection is also affected (18).

**Dephosphorylation of SR Proteins and alternative splicing of caspase 9**

In addition, the generation of endogenous ceramide and PP1 activation was also shown to mediate the alternative splicing of an apoptotic factor, caspase 9, in a manner that promotes apoptosis. Ceramide treatment resulted in a decrease in the processing and expression of the pro-survival caspase 9b isoform of caspase 9 with a concomitant increase in the pro-apoptotic caspase 9a isoform. This effect required the generation of endogenous ceramide through the *de novo* pathway, and more importantly, inhibitors of protein phosphatase-1 abolished the ability of ceramide to affect the alternative splicing of caspase 9. A hypothesis linking *de novo* ceramide synthesis to the alternative splicing of caspase 9 is shown in figure 5. Interestingly, it has been found that increased expression of caspase 9a due to *de novo* ceramide production occurred in PP1-dependent manner. Thus, both the phospho-state of SR proteins and the alternative splicing of caspase 9 are regulated by the generation of *de novo* ceramide and subsequent PP1 activation. These data suggest that alternative splicing may be an important mechanism for the regulation of apoptosis by ceramide.
The involvement of PP1 and endogenous ceramide in the dephosphorylation of SR proteins and the effects on caspase 9 alternative splicing suggested that at least one SR protein isoform was involved in this pathway. Previous studies using siRNA to SRp30a, in A549 lung adenocarcinoma cells showed that SRp30a is a major regulator in the alternative splicing of caspase 9 as shown in figure 6. A549 lung adenocarcinoma cells were treated with 200 nM siSRp30a and western blot analysis revealed an approximate 85% downregulation of SRp30a (figure 6). RT-PCR analysis revealed a dramatic decrease in the caspase 9a/caspase 9b ratio. In these studies, we identified five possible purine-rich RNA sequences within the caspase 9 gene that correspond to the binding sites of SRp30a, and function as RNA cis-elements in the alternative splicing of caspase 9.
Figure 5. Hypothesis of the signal transduction pathway mediating the alternative splicing of caspase 9. Chemotherapeutics act on A549 cells leading to de novo ceramide synthesis. This in turn leads to the activation of protein phosphatase 1 (PP1), which dephosphorylates SR proteins. Dephosphorylated SRp30a, an SR protein family member, binds to purine-rich cis-elements of the exon 3, 4, 5, 6 cassette of caspase 9 pre-mRNA in order to regulate inclusion and exclusion of that cassette and favoring the caspase 9a splice variant.
Figure 6. RNAi used to down regulate SRp30a. RNAi technology was used to downregulate SRp30a. A549 lung adenocarcinoma cells were treated with 200 nM siSRp30a, and Western blot analysis revealed an approximate 85% downregulation of SRp30a (right panel). RT-PCR analysis revealed a dramatic change in the caspase 9a/caspase 9b ratio (left panel). These studies were performed by Autumn Massiello of the Chalfant laboratory.
CHAPTER 2 Materials and Methods

Cell Culture: A549 lung adenocarcinoma cells were purchased from American Type Tissue Culture Collection (http://www.atcc.org). These A549 cells were grown in 50% RPMI 1640 (Invitrogen) and 50% DMEM (Invitrogen) supplemented with L-glutamine, 10% (v/v) fetal bovine serum (Invitrogen), 100 U/ml of penicillin G sodium (Invitrogen), and 100 µg/ml of streptomycin sulfate (Invitrogen). Cells were maintained at less than 80% confluency under standard incubator conditions (humidified atmosphere, 95% air, 5% CO₂, 37°C).

Electromobility Shift Assay: The following sequences were used for FITC-tagged RNA oligonucleotides: In Exon 3: r(5’Fl- gaguguuggggaau), Between Exon 4 and Exon 5: r(5’Fl- uggaggaccaugggaggua) and in Exon 6: r(5’Fl- uggagggagaugcccagcu). The following sequences were used for RNA oligonucleotide competitors without fluorescein: In Exon 3: r(5’-ugaggggaau), Between Exon 4 and Exon 5: r(5’- augggaggu) and in Exon 6: r(5’- ugggagggaagc). RNA binding reactions were performed in a final volume of 25 µl containing: 100 ng of fluorescein-oligonucleotide, 50 µg nuclear protein extract or 50 µg of recombinant SRp30a (generously provided by Dr. Adrian Krainer, Cold Harbor Springs Laboratory, New York), 40 U RNASIN, and 11.3 µg tRNAs in buffer composed of 50 mM HEPES, 5 mM DTT, 625 mM KCl, 5 mM DTT, 5 mM EDTA, and 0.125%
NP40. The reaction mixtures were incubated at 25°C for 30 minutes and then crosslinked with ultraviolet light for 30 minutes. Half of these samples were loaded on a 5% TBE Criterion™ Precast Gel (BioRad) for electrophoretic separation of RNA-protein(s) complexes for 1.5 hours at 115 volts at 4°C. The gel was then scanned using Molecular Imager® FX (BioRad) with a 488nm EX (530 nm BP) laser. The Molecular Imager® FX (BioRad), performs densitometry analysis and reveals differences in a given scan only within the linear range of statistical significance. An equal volume of 2 x Laemmli buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.04% bromophenol blue, and 250 mM β-mercaptoethanol) was added to the other half of the samples which were boiled for 10 minutes. These samples were run on a 10% Tris-HCL Criterion™ Precast Gel (BioRad) for electrophoretic separation of RNA-protein(s) complexes for 1.5 hours at 50 volts through the stacking gel and then at 100 volts at 25°C. For supershift reactions, antibody to SRp30a (provided by Dr. Adrian Krainer, Cold Harbor Springs Laboratory, New York (1:10 diluted)) or non-specific IgG (1 mg/mL and 1:10 diluted (Sigma)) was added 20 min after addition of the oligonucleotide with an additional 30 min of binding time at 4°C. Samples were then subjected to electrophoretic separation and analyzed as described above.

Reverse Transcription Polymerase Chain Reaction (RT-PCR) Assay: Total RNA from A549 cells was isolated using the RNeasy kit (Qiagen) per manufacturer’s protocol. This kit allows the investigator to lyse cells and remove all cellular components including lipids, proteins, and DNA and purify only RNA. This is done in an RNase free
environment. 1 µg of A549 total RNA was reverse transcribed using Superscript II reverse transcriptase (Invitrogen) and oligo(dT) as the priming agent. After 50 min incubation at 42 °C, the reactions were stopped by 70° C heating for 15 min. Template RNA was then removed using RNase H (Invitrogen).

For evaluating the expression of caspase 9 splice variants derived from the minigene, an upstream vector 5' primer to caspase 9 (5' TCACTATAGGGAGACCCAAGC) and a 3' primer E7A (5' AAGATGTCACTGGGTGTGGGCAAACTAGA) (Genosys, Inc.) were used. Using these primers, 20% of the reverse transcriptase reaction was amplified for 35 cycles (94°C, 30s melt; 63°C, 30 s anneal; 72°C, 1 min extension) using Platinum Taq DNA polymerase (Invitrogen). The RT-PCR reaction was examined by 1.5% agarose gel electrophoresis and scanned on a Molecular Imager® FX (BioRad). The Molecular Imager® FX (BioRad), performs densitometry analysis and reveals differences in a given scan only within the linear range of statistical significance.

Nuclear Extracts: Nuclear extracts were prepared from A549 lung adenocarcinoma cells according to the method of Dignam et al. (11) by Autumn Massiello. Protein concentrations were determined by a modification of the Bradford method using the Bio-Rad protein assay reagent (Bio-Rad).

Protein Extraction: Cells were lysed with 0.1 mL of 2 x Laemml buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.04% bromophenol blue, and 250 mM β-mercaptoethanol) after resuspension in 0.1 mL of ice-cold phosphate-buffered saline (PBS). Samples were
boiled for 10 min and either examined directly by 10% SDS-polyacrylamide gel electrophoresis or stored at -20°C.

*Western Immunoblotting:* Total protein lysate (20 μg) was subjected to 10% SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membrane (Bio-Rad) and blocked in 5% milk, 1 × PBS-T (M-PBS-T) for 2 h. The membrane was incubated with anti-SRp30a (provided by Dr. Adrian Krainer, Cold Harbor Springs Laboratory, New York (1:100 diluted)) or anti-α-tubulin (Santa Cruz Biotechnology (1:2000 diluted)) for 2 h in M-PBS-T followed by 3 washes with PBS-T. The membrane was then incubated with a secondary antibody of horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (anti-SRp30a) or horseradish peroxidase-conjugated anti-mouse IgM (Cal-Biochem) (anti-α-tubulin) for 45 min followed by 3 washes with PBS-T. Immunoblots were developed using Pierce ECL reagents and Bio-Max film.

*Replacement Mutagenesis:* The caspase 9 minigene was cloned into the vector pcDNA3.1(-) Zeocin purchased from Invitrogen. The five sequences selected for replacement mutagenesis are shown in figure 7. To mutate these purine-rich sequences in Exon 3, between Exons 4 & 5, between Exons 5 & 6, Exon 6, and Exon 6 & 7, the Quikchange II XL Site-Directed Mutagenesis Kit by Stratagene was used. This kit enables the investigator to introduce mutations into a cloned gene by several techniques. First, the kit provides instructions on primer design with regards to % mismatch, length, and GC content. Secondly, it provides the reagents for mutant strand synthesis, digestion of parental strand, and the subsequent transformation of bacterial cells. To mutate the selected
purine-rich sequences in the caspase 9 minigene and for efficient screening of mutants, these purine-rich sequences were mutated to Hind III sites. The mutagenesis primers used for the Exon 3 mutant are 5’ CAGGTGCTCTTGAGAGTTTGAAAGCTTGATGCAGATTTGGTGAG 3’ and 3’GTCCACGAGAACTCTCAAACCTTCGAAACTACGTCTAAACCACTC 5’.

The mutagenesis primers used for the Exon 4 and 5 mutant are 5’ GCCCTTACATCTGGAGGACCATAAAGCTTTAGGACGCGCCCCAGCGGC 3’ and 3’ CCGGAATGTAGACCTCTCTGTATCTCGAAATCTCTGCCGGGTCGCCG 5’.

The mutagenesis primers used for the Exon 5 and 6 mutant are 5’ CTGAGGAGAAGCCCTCTGGGAGAAGCTTTGCGCAGAGGCTCTGC 3’ and 3’ GACCCTCTTCTCGGAGACCCTCTCTCGAAAGTCTCTTGTCAGACG 5’.

The mutagenesis primers used for the Exon 6 mutant are 5’ CCAGCTGCCCCAGCCTGGGAAAGCTTTCCCAAGCTCTTTTTTCATCC 3’ and 3’ GGTCGACGCGGTGTCAGCCCTCTCGAAAGGTTCAGAGAAAAAGGTAGG 5’.

The mutagenesis primers used for the Exon 6 and 7 mutant are 5’ GGGTTGGGTCTGGTGGGGAAGCTTCCGACCACCTGCTTC 3’ and 3’ CCCACCCAGACCCACCACCCTTCGAAAGGCCTGGACGAAG 5’.

The resulting plasmids were purified to be endotoxin free using the services of the Molecular Biology Core Facility of the Massey Cancer Center of Virginia Commonwealth University.
Figure 7. The five purine-rich sequences of capase 9 pre-mRNA to undergo replacement mutagenesis.

<table>
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<th>Sequence</th>
<th>Purine-Rich SRp30a Binding Sequence</th>
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<tr>
<td>1.........</td>
<td>aggggaa</td>
<td>Exon 3 (18,333-18,339 bp)</td>
</tr>
<tr>
<td>2.........</td>
<td>gggagg</td>
<td>Btw Exons 4&amp;5 (19,441-19,446 bp)</td>
</tr>
<tr>
<td>3.........</td>
<td>agggagg</td>
<td>Btw Exons 5&amp;6 (20472 – 20478 bp)</td>
</tr>
<tr>
<td>4.........</td>
<td>agggaag</td>
<td>Exon 6 (21,582-21,588 bp)</td>
</tr>
<tr>
<td>5.........</td>
<td>agggag</td>
<td>Btw Exon 6&amp;7 (21648-21653 bp)</td>
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</table>

Exon 3,4,5,6 Cassette

18,326 bp

21,622 bp
Sequence Verification of Minigene Splice Variants: In order to ensure that the sequences of the splice variants of the caspase 9 minigene correspond to the actual sequences of the caspase 9a and 9b splice variants minus Exon 1, Exons 8, and Exon 9, the cloned caspase 9 minigene (figure 8) was transfected into A549 lung adenocarcinoma cells. After 24 hours, RNA was isolated from the cells using Qiagen’s RNeasy Mini Kit (described previously). RT-PCR was performed as outlined above. Once the PCR reactions were examined by 1.5% agarose gel electrophoresis, bands on the gel suspected of being the caspase 9a variant and the caspase 9b variant were removed from the gel, and the RT-PCR products were isolated using the Qiagen MinElute Gel Extraction Kit. This kit allows the investigator to isolate DNA from an agarose gel by dissolving the agarose and eluting it on a column followed by the elution of purified DNA. These RT-PCR products were sent to SeqWright DNA Technology Services to be sequenced and their sequences were verified by checking their exon sequences against the NCBI database found at http://www.ncbi.nlm.nih.gov/.
1. Step 1: High Fidelity PCR of the Genomic Gene Pieces of Caspase 9

2. High Fidelity PCR of Caspase 9 PCR fragments with Restriction Sites.

**Figure 8. Structure and cloning of the caspase 9 minigene.**

The caspase 9 minigene was constructed by amplifying selected gene pieces of the caspase 9 gene using genomic DNA. This resulted in a caspase 9 minigene that excluded exon 1, exon 8, and exon 9. 2) Restriction sites were then engineered into the caspase 9 minigene so that when the caspase 9 minigene was digested with the corresponding restriction enzymes and the vector used for cloning (pcDNA3.1/Zeo) was digested with these same enzymes, the caspase 9 minigene could be cloned into this vector and subsequently used for transfections.
CHAPTER 3 Results

Caspase 9 Minigene producing splice ratio similar to that of endogenous caspase 9

Previously, a minigene plasmid to examine the alternative splicing of caspase 9 in cells was constructed (figure 8). To examine whether this construct behaved in a similar manner as the endogenous gene, the plasmid was transiently transfected into A549 cells. Using an RT-PCR based assay that examines only the minigene produced RNA, the ratio of minigene caspase 9a/9b was determined to be 3.0, comparable to the endogenous ratio of 2.0. The resulting RT-PCR products were visualized using agarose gel electrophoresis (figure 9).
Figure 9. Transfection of A549s with the caspase 9 minigene (N=3). After transfection with the caspase 9 minigene, the wild type minigene produced the caspase 9a (910 BP) and caspase 9b (446 BP) splice variants in a ratio (approximately 3:1) analogous to that of the endogenous wild type caspase 9 gene (approximately 2:1). These caspase 9 minigene products were cloned into a TOPO vector and sequence verified.
Figure 10. Sequence Verification of the caspase 9 splice variants produced by the caspase 9 minigene. Shown above are the sequences of the isolated RT-PCR products of caspase 9a and 9b. After obtaining the these sequences, they were verified using the NCBI database as the exact sequences of the endogenous caspase 9a splice variant and caspase 9b variant minus exons 1, 8, and 9 and containing the expected pcDNA3.1(-) Zeocin sequence.
Sequence Verification of the caspase 9 splice variants produced by the caspase 9 minigene

The PCR products of the splice variants caspase 9a and caspase 9b shown in figure 9 were removed from the agarose gel, cloned into a pBlueBac4.5/V5-His-TOPO vector from Invitrogen, and sequenced using SeqWright DNA Technology Services. Figure 10 shows that the caspase 9 splice variants produced by the caspase 9 minigene are the exact sequences of the endogenous caspase 9a splice variant and caspase 9b variant minus exons 1, 8, and 9 and containing the expected pcDNA3.1(-) Zeocin sequence.

Replacement Mutagenesis of Sequences 1, 2, and 4 alters the splice ratio of caspase 9a and 9b

Replacement mutagenesis was performed on five purine-rich sequences (Sequences 1-5 shown in figure 7) in the caspase 9 minigene as outlined in the Materials and Methods section. Of these five sequences, three of them, when mutated, affected the splice ratio of caspase 9a and caspase 9b. Visualized in figure 11 are the RT-PCR products from A549 cells transfected with caspase 9 minigene with two different mutations introduced into sequences 2 and 4. The mutation of these sequences corresponding to the purine-rich sequence between Exons 4 and 5 and the purine-rich sequence in Exon 6, induced a decrease in the splice ratio of caspase 9a/caspase 9b. Therefore, when both sequences 2 and 4 are mutated, a decrease in the splice ratio of caspase 9a/caspase 9b is observed.

Visualized in figure 12 are the RT-PCR products from A549 cells transfected with caspase 9 minigene with sequence 1 mutated. Mutating this sequence increased the splice
ratio of caspase 9a/caspase 9b. Since this mutation results in an increase of the caspase 9a/caspase9b ratio, this purine-rich sequence in Exon 3 acts as a repressor element. Mutagenesis of sequences 3 and 5 had no effect on the ratio of caspase 9a/caspase 9b.
Figure 11. Purine-Rich RNA Cis-Elements #2 and #4 Affect the Alternative Splicing of Caspase 9 Pre-mRNA (N=6). 1= 100 BP ladder, 2= Vector Control, 3= Wild Type Caspase 9 Minigene, 4= E6 Mutant 4 minigene, 5= E4 and E5 Mutant 2 minigene. These two mutants shift the direction of the splice ratio toward caspase 9b.
Figure 12. Purine-Rich RNA *Cis*-Element #1 Acts As A Repressor Element for the Caspase 9a Splice Variant (N=6). 1= 100 BP ladder, 2= Vector Control, 3= Wild Type C9 Minigene, 4= E3 Mutant 1 minigene. After mutating this purine-rich sequence in exon 3 the splice ratio shifts in the direction of caspase 9a indicating that it acts as a repressor element.
RNA/Protein Binding Studies Using Electromobility Shifts and SDS PAGE

After the replacement mutagenesis on the five purine-rich sequences yielded three sequences that were possible sites for binding RNA splicing factors, fluorescein-tagged oligonucleotides corresponding to these three sequences were used in electromobility shift assays and SDS-PAGE assays. Since the purine-rich sequence in Exon 3 was a possible repressor element in caspase 9 pre-mRNA, the corresponding fluorescein tagged oligonucleotide and its competitive inhibitor were used in EMSA assays to examine if this oligonucleotide bound proteins from nuclear extracts, which are rich in transcription factors and splicing factors. The EMSA competitor study in figure 13 showed that the fluorescein-tagged exon 3 sequence bound RNA trans-factors and is effectively inhibited by the addition of an unlabeled oligonucleotide corresponding to the purine-rich exon 3. Thus, this sequence is a possible candidate for the binding of a repressor protein.

Those assays were repeated and subjected to SDS-PAGE analysis for determination of the specific protein (or protein complex) size bound to the RNA sequence as shown in figure 14. The RNA oligonucleotides used for this assay correspond to the purine-rich sequences in sequence 1 and sequence 4. These results demonstrate the specific binding of ~66, 56, 45, 38 kDa proteins to the oligonucleotide sequence in exon 3 and an ~66 kDa protein(s) bound to the exon 6 RNA oligonucleotide sequence.
Figure 13. EMSA Competitor Studies for Sequence 1(Exon 3) Using A549 Nuclear Extracts (N=3). Lane 1- EMSA as described previously, including nonspecific competitor oligo- gaauugcauguua. Lane 2- EMSA as described previously, including specific competitor oligo for exon 3 – ugggaggaagcc. The competition away in lane 2 shows that the purine-rich sequence in exon 3 binds proteins in the A549 nuclear extract.
Figure 14. SDS-PAGE Analysis To Determine Sizes of Protein Complexes bound to RNA sequences (N=3). NSC= Nonspecific Competitor and SC= Specific Competitor. These results demonstrate the specific binding of ~66, 56, 45, 38 kDa proteins to the oligonucleotide sequence in exon 3 (possible repressor element).
Figure 15. EMSA and SDS-PAGE Competitor Studies For Sequence 2 and 4 (Intron 4-5 and Exon 6) Using A549 Nuclear Extracts (N=4). Lane 1- EMSA as described previously, including nonspecific competitor oligo- gaauugcagguua (NSC), Lane 2- EMSA as described previously, including specific competitor oligo (SC). Introduction of specific competitors and the subsequent losses of shift are indicative of these sequences binding to proteins in A549 nuclear extract.
Because the mutation in sequence 2 (Intron 4-5) shifted the ratio of the splice variants in the direction of caspase 9b, the EMSA competitor studies and SDS-PAGE was repeated using a fluorescein-tagged oligonucleotide corresponding to that sequence. In figure 15, the EMSA and SDS-PAGE competitor studies showed that fluorescein-tagged oligonucleotides corresponding to the purine-rich sequences in sequence 4 and sequence 2 bind specifically to RNA trans-factors. This was confirmed by competition studies. Thus, sequence 2 binds trans-factors of sizes 66, 56, and 38 Kd, and sequence 4 also binds trans-factor complexes of 66, 56, and 38 kD in size.

We hypothesized that SRp30a might bind sequence 2 and sequence 4 given that the literature shows SRp30a having a strong affinity for purine-rich sequences. Thus, it was important to establish whether or not SRp30a interacts with these sequences. Recombinant SRp30a was obtained from a collaborating lab, and these EMSAs were repeated using this recombinant SRp30a. As seen in figure 16, when the EMSA was repeated using recombinant SRp30a, sequences 2 and 4 bound to SRp30a, indicating that these sequences have a strong affinity for SRp30a.
Figure 16. EMSA Competitor Studies For Sequence 2 and 4 (Intron 4-5 and Exon 6) Using Recombinant SRp30a (N=3). 1= Control (IgG), 2= Intron 4-5 Oligo, 3= Exon 6 Oligo. In the control lane, there is no shift evident. In lanes 2 and 3, a clear shift can be seen when recombinant SRp30a is used which shows the strong affinity of SRp30a for these two purine-rich sequences. (These experiments were conducted in collaboration with Autumn Massiello of the Chalfant laboratory).
Additional confirmation that SRp30a bound to these sequences was done by repeating the EMSA using both A549 nuclear extracts and recombinant SRp30a and the inclusion of an antibody specific to SRp30a. In figure 17, addition of an antibody specific to SRp30a induced a “supershift” with a concomitantly decrease of SRp30a/sequence 4 complex. This confirmed that SRp30a bound to these two purine-rich sequences that regulate the inclusion of the exon 3,4,5,6 cassette of the caspase 9 gene.
Figure 17. EMSA Supershift for Sequence 4 (Exon 6 oligo) (N=3). The first two lanes are EMSAs using recombinant SRp30a and the last two lanes are EMSAs using A549 nuclear extracts. Non-specific IgG antibody is used as a control for these EMSAs, and IgG antibody specific to SRp30a is used to create both loss of shift and a supershift indicating that SRp30a does bind to this purine-rich sequence (These experiments were conducted in collaboration with Autumn Massiello of the Chalfant laboratory).
Chapter 4 Discussion

Previously, our laboratory showed that siRNA to SRp30a changed the splice ratio of caspase 9a/9b showing a decrease in caspase 9a expression with a concomitant increase in caspase 9b expression. In this study, we showed that mutation of the purine-rich sequences 2 and 4 (Intron 4-5 and Exon 6 of the caspase 9 gene) induced a decrease in the splicing ratio of caspase 9a/caspase 9b. This finding demonstrated that these RNA cis-elements are required for the inclusion of the exon 3, 4, 5, 6 cassette and are exonic splicing enhancers. Furthermore, mutation of the purine-rich sequence in exon 3 induced an increase in the caspase 9a/caspase 9b ratio acting as a repressor RNA cis-element. Both exonic enhancers specifically bound RNA splicing factors, specifically SRp30a. These findings are important for several reasons. First, the alternative splicing mechanism of caspase 9 splicing is better defined. Second, these findings demonstrate a link between SR proteins and the alternative splicing of caspase 9. Finally, these findings have direct relevance to mechanisms governing the sensitization of cancer cells to chemotherapy.

Liu et al previously reported that SRp30a had an affinity for the purine-rich sequences, RGAACAAC and AGGACRRAGC, based on SELEX binding studies where R represents a purine (12). In additional studies they performed, they found exon splicing enhancers (ESEs) with sequences, AGGAGGA, GACCCGG, CGCAGGG, CAGAGGU, and GAGACGA bound SRp30a with high affinity and greatly enhanced splicing (12). Furthermore, unpublished studies from our laboratory showed that siRNA against SRp30a decreased the splice ratio of caspase 9a/caspase 9b. Based on these findings, we proceeded
with replacement mutagenesis of five sites identified in caspase 9 pre-mRNA that are as uncharacteristically purine-rich as the aforementioned sequences. Indeed, mutating sequences 2 and 4 had a dramatic effect on the alternative splicing of caspase 9, reducing the ratio of caspase 9a/caspase 9b. Binding studies using oligonucleotides that correspond to these sequences demonstrated specific interaction of these sequences with RNA trans-factors. Our laboratory further showed that these sequences interacted specifically with SRp30a. This finding was important because it provided strong mechanistic evidence that SRp30a plays an important role in alternative splicing of caspase 9. Thus, for the first time, SRp30a was shown to be involved in regulating the inclusion of a large exon cassette as is the case for caspase 9 pre-mRNA.

Given these findings, there are finally implications for a link between de novo ceramide synthesis and the dephosphorylation of SR proteins favoring to the caspase 9a phenotype. Although there is no current evidence directly linking dephosphorylation of SR proteins to the alternative splicing of caspase 9, the SR protein SRp30a, is now directly linked as an important regulator of this mechanism. As mentioned before, PP1 has been reported as a target for activation by natural ceramides in vitro (9), and SR proteins have been shown to be substrates of PP1 (9). Chalfant et al have reported that de novo ceramide synthesis increases the ratio of caspase 9a/caspase 9b and in their experiments, inhibitors to PP1 abolished this effect of ceramide on the splice ratio, thus showing that ceramide altered the splice ratio in a PP1 dependent manner (13). Because our laboratory showed that siRNA against SRp30a decreased the splice ratio of caspase 9a/caspase 9b and SR
proteins are known substrates of PP1, the accumulated indirect evidence strongly suggests a link between de novo ceramide synthesis and the dephosphorylation of SRp30a leading to an increase in the splice ratio of caspase 9a/caspase 9b.

This leads us to a possible future target for therapy in lung adenocarcinoma cells. If excessive phosphorylation of SRp30a in the spliceosome leads to an anti-apoptotic(pro-cancer phenotype) then a possible target could be the kinase that phosphorylates SRp30a. Prasad et. al have reported that Clk/Sty (Clk1) is a protein kinase that when autophosphorylated, phosphorylates SR proteins (14). Which SR proteins get phosphorylated by Clk/Sty depends on the nature of the autophosphorylation that it undergoes. From their experiments, they have found that when Clk/Sty autophosphorylates on Tyr residues, but not Ser/Thr residues, the result is phosphorylation of SRp30a (14). Interestingly, they found that SC35 is phosphorylated when Ser/Thr residues are autophosphorylated by Clk/Sty, and that SRp40 is phosphorylated by Clk/Sty when no autophosphorylation takes place (14). It is evident that Clk/Sty is responsible for phosphorylating several different SR proteins depending on its autophosphorylation status, so this allows for different scenarios of targeted therapy. One possibility would be to treat cells with antibodies specific to Clk/Sty, which are tagged with catalytic residues that alter the tyrosine phosphorylation of Clk/Sty. This would be a precise targeting of Clk/Sty that would allow it to function as a kinase for all its other substrates except for SRp30a. Perhaps this targeted therapy scheme seems too unrealistic, so a more reasonable approach to therapy would be to introduce to adenocarcinoma cells siRNA to Clk/Sty to
downregulate it, so that SRp30a would be less phosphorylated, and the catalytic activity of PP1 on SRp30a would be more effective in yielding the pro-apoptotic caspase 9a phenotype. Even this kind of treatment must be monitored very closely, because as mentioned before, Clk/Sty phosphorylates other SR proteins as well including SRp40 and SC35. It is quite possible that excessive dephosphorylation of these SR proteins could have undesirable consequences. Lastly, since we have found that sequence 1 (in Exon 3) acts as repressor element, which when mutated, favors the pro-apoptotic phenotype, adenocarcinoma cells could be treated locally by transfecting them with a cloned full caspase 9 gene with a silent mutation introduced in this purine-rich repressor sequence. The silent mutation would allow for the coding of the same amino acids, but would not be purine-rich. In this way, we could shift the splice ratio in the direction of caspase 9a while still having a completely functional caspase 9a.

So, preliminary studies have shown that siRNA to SRp30a decreased the splice ratio of caspase 9a/9b. Furthermore, when the purine-rich binding sites for SRp30a in Intron 4-5 and Exon 6 of the caspase 9 gene are mutated, there is also a decrease in the splice ratio of caspase 9a/caspase 9b. Because of these findings we realize now that SR proteins, specifically SRp30a, are part of the mechanism of the alternative splicing of caspase 9 and that SRp30a fits into the cascade of de novo ceramide synthesis leading to the alternative splicing of caspase 9. Finally, these findings allow for the possibility of new types of chemotherapy to treat cancer.
Therefore in this study, we have identified two purine-rich sequences that act as exonic splicing enhancers for the inclusion of the exon 3, 4, 5, 6 cassette of caspase 9 pre-mRNA. The Chalfant laboratory demonstrated that these ESE's interact specifically with SRp30a. Therefore, the finding that SRp30a is part of the mechanism of the alternative splicing of caspase 9 pre-mRNA finally provides the critical link to PP1 and \textit{de novo} ceramide synthesis. Lastly, these findings allow for the possibility of new types of chemotherapy to treat cancer as well as possible diagnostic applications.
Literature Cited


Prabhat Mukerjee was born in Iowa City, Iowa in 1973. He grew up in Philadelphia, Pennsylvania where he graduated from the Episcopal Academy in 1991. He completed his Bachelor of Science degree in Biochemistry from Bucknell University in 1995. He proceeded to work in the business world, starting in sales, and then moved on to writing software for financial systems. He joined the graduate program at The Medical College of Virginia in the fall of 2003 and defended his Master of Science thesis in the summer of 2005. He will be matriculating to the M.D. program at The Medical College of Virginia in the fall of 2005.