SRp30a (ASF/SF2) regulates the alternative splicing of caspase-9 pre-mRNA and is required for ceramide-responsiveness

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Abstract Two splice variants are derived from the caspase-9 gene, proapoptotic caspase-9a and antiapoptotic caspase-9b, by either the inclusion or exclusion of an exon 3, 4, 5, and 6 cassette. Previous studies from our laboratory have shown that the alternative splicing of caspase-9 and the phosphorylation status of SR proteins, a conserved family of splicing factors, are regulated by chemotherapy and ceramide via the action of protein phosphatase-1. In this study, a link between ceramide, SR proteins, and the alternative splicing of caspase-9 was established. The downregulation of SRp30a in A549 cells by RNA interference technology resulted in an increase in the caspase-9b splice variant, with a concomitant decrease in the caspase-9a splice variant, thereby significantly decreasing the caspase-9a/9b ratio from 1.67 ± 0.11 to 0.56 ± 0.08 (P < 0.005). The specific downregulation of SRp30a also inhibited the ability of exogenous ceramide treatment to induce the inclusion of the exon 3, 4, 5, and 6 cassette. Therefore, we have identified SRp30a as an RNA trans-acting factor that functions as a major regulator of caspase-9 pre-mRNA processing and is required for ceramide-responsiveness.

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Supplementary key words RNA cis-element • RNA trans-acting factor • A549 cells

Ceramide is an important regulator of various stress responses and growth mechanisms, 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, 1a,25-dihydroxyvitamin D₃, interleukin-1, ultraviolet light, heat, chemotherapeutic agents, fatty acid synthase antigen, and nerve growth factor (1–7). Also, the addition of exogenous ceramide or the enhancement of cellular levels of ceramide induces cell differentiation, cell cycle arrest, apoptosis, or cell senescence in various cell types (8–10). The prominent role of ceramide as a regulator of cellular mechanisms necessitated the identification of target molecules. To this end, a family of ceramide-regulated enzymes has been identified, ceramide-activated protein phosphatases, which include the serine/threonine-specific protein phosphatases PP1 and PP2A (11–14). With the demonstration of PP1 as a ceramide-activated protein phosphatase, potential PP1 substrates and mechanisms regulated by PP1 became candidate targets for ceramide action.

SR proteins, a family of arginine/serine-rich domain-containing proteins and specific PP1 substrates, are required for constitutive and alternative pre-mRNA processing. Endogenous ceramide was recently found to modulate the phosphorylation status of SR proteins in a PP1-dependent manner (15). Several reports have also demonstrated a role for PP1 in regulating alternative splicing, and two splicingosomal targeting subunits for PP1 have been described (16–19). Therefore, PP1 may play a role in regulating RNA processing in response to apoptotic stimuli; in particular, it may define a pathway linking ceramide to the regulation of the alternative splicing of apoptotic regulators.

To this end, our laboratory recently described a pathway linking the generation of de novo ceramide and the activation of PP1 to the regulation of the exclusion or inclusion of an exon 3, 4, 5, and 6 cassette of caspase-9 pre-mRNA (20). Ceramide treatment resulted in an increase in proapoptotic caspase-9a (cassette inclusion) mRNA and protein levels and a concomitant decrease in caspase-9b (cassette exclusion) mRNA and protein levels in A549 cells (20). This effect required the generation of endogenous ceramide through the de novo pathway; more importantly, inhibitors of PP1 abolished the ability of ceramide to affect...
the alternative splicing of caspase-9 (20). Thus, both the phosphorylation state of SR proteins and the alternative splicing of caspase-9 are regulated by the generation of de novo ceramide and subsequent PP1 activation (15, 20).

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 regulated the alternative splicing mechanism of caspase-9. In this study, we identified an SR protein, SRp30a, as a critical splicing factor in the alternative splicing of caspase-9 pre-mRNA. Furthermore, we demonstrated that SRp30a is a required RNA trans-acting factor for ceramide to affect the alternative splicing of caspase-9 pre-mRNA, thereby linking de novo ceramide generation, SRp30a, and the regulation of caspase-9 expression.

EXPERIMENTAL PROCEDURES

Cell culture
A549 adenocarcinoma cells were grown in 50% RPMI 1640 (Invitrogen) and 50% DMEM (Invitrogen) supplemented with t-glutamine, 10% (v/v) fetal bovine serum (Invitrogen), 100 U/ml penicillin G sodium (Invitrogen), and 100 µg/ml streptomycin sulfate (Invitrogen). Cells were maintained at 95% air, 5% CO2, under standard incubator conditions (humidified atmosphere, 37°C).

Small interfering RNA transfection
Transfection of A549 cells with the SRp30a SMARTpool® or SRp30a SMARTselection™ designed silencer RNA (siRNA) reagents (Dharmacon) was performed using Oligofectamine (Invitrogen) according to the manufacturer’s (Invitrogen) protocol. The duplex RNA targeting sequences used were as follows: SRp30a-1 sense sequence (5'-GAA AGA UAU GAC CUA UU-3') and antisense sequence (5'-AUA GGU CAU AUC UUG UU-3'); SRp30a-2 sense sequence (5'-UAA CGC UCC AGG AGA CAU CUA UU-3') and antisense sequence (5'-GAU AGG UGG AGG UAU GUU AUC-3'); SRp30a-3 sense sequence (5'-UGA AGC AGG UGA UGU AUG UU-3') and antisense sequence (5'-ACA UAC AUC ACC UGC UUC AUC UU-3'); and SRp30a-4 sense sequence (5'-CGA CGG CUA UGA UUA CGA UU-3') and antisense sequence (5'-ACG UAU AUC AUA GCC GUC GUU-3'). Briefly, A549 cells were plated in regular growth medium at 40–50% confluence in a six-well tissue culture dish 24 h before transfection. Cells in 1.8 ml of Opti-Mem® I medium without antibiotics/fetal bovine serum were transfected with 200 nM (dilution in Opti-Mem® I) of the oligonucleotide (in 15 µl of 4 µl of Oligofectamine/Opti-Mem® I reduced serum medium) and incubated for 4 h in standard incubator conditions. After incubation, 0.5 ml of Opti-Mem® I reduced serum medium containing three times the normal concentration of antibiotics/fetal bovine serum was added to the transfected A549 cells without removing the transfection mixture. After 48 h, total RNA or total protein lysate was collected as described below for RT-PCR or Western blot analysis.

Western immunoblotting
Total protein lysate (20 µg) was subjected to 10% SDS-PAGE. Proteins were transferred to a polyvinylidene difluoride membrane (Bio-Rad) and blocked in 5% milk and 1× PBS-Tween (M-PBS-T) for 2 h. The membrane was incubated with anti-SRp30a or anti-a-tubulin for 2 h in M-PBS-T followed by three washes with PBS-T. The membrane was then incubated with a secondary antibody of horseradish peroxidase-conjugated goat anti-mouse IgG antibody (Pierce; anti-SRp30a) or horseradish peroxidase-conjugated anti-mouse IgM (Calbiochem; anti-a-tubulin) for 45 min followed by three washes with PBS-T. Immunoblots were developed using Pierce ECL reagents and Bio-Max film.

RT-PCR
Total RNA from A549 cells was isolated using the RNeasy kit (Qiagen) according to the manufacturer’s protocol. One microgram of A549 total RNA was reverse-transcribed using Superscript II reverse transcriptase (Invitrogen) and oligo(dT) as the priming agent. After 50 min of incubation at 42°C, the reactions were stopped by heating at 70°C for 15 min. Template RNA was then removed using RNase H (Invitrogen). To evaluate the expression of endogenous caspase-9 splice variants, an upstream 5’ primer to caspase-9 (5’-GGT CTT CTT TTG TTC ATC TGC 3’) and a 3’ primer (5’-CAT CTG GCT CGG GGT TAC TGC 3’) (Integrated DNA Technologies, Inc.) were used. Using these primers, 20% of the reverse transcriptase reaction was amplified for 35 cycles (94°C, 30 s melt; 58°C, 30 s anneal; 72°C, 1 min extension) using Platinum Taq DNA polymerase (Invitrogen). The PCR product was examined by 1.5% agarose gel electrophoresis. The gel was then stained with SYBR® Gold (Invitrogen) and scanned using a Molecular Imager® FX (Bio-Rad) with a 488 nm EX (530 nm BYPASS) laser.

Protein extraction
Total protein was extracted by direct lysis with Laemmli buffer. Cells were lysed with 0.1 ml of 2× Laemmli buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.04% bromphenol blue, and 250 mM β-mercaptoethanol) after resuspension in 0.1 ml of ice-cold PBS. Samples were boiled for 10 min and either examined directly by SDS-PAGE or stored at −20°C.

RESULTS
SRp30a regulates the alternative splicing of caspase-9
SR proteins are well-established regulators of the exon inclusion/exclusion of alternative splicing mechanisms (21–24). Based on previous findings from our laboratory demonstrating that PP1 activation by de novo ceramide generation can regulate both the phosphorylation status of SR protein and the pre-mRNA processing of caspase-9 (15, 20), we hypothesized that an SR protein isoform is involved in the regulation of the alternative splicing mechanism of caspase-9 pre-mRNA. To determine whether an SR protein played a role in regulating the alternative splicing of the caspase-9 pre-mRNA transcript, RNA interference (RNAi) technology was used to downregulate several SR family proteins as well as other splicing factors suggested in the literature to regulate apoptosis and the alternative splicing of the closely related enzyme, caspase-2 (25–32) (Table 1). A “pool” of siRNA targeting SRp30a resulted in an ~80% downregulation of SRp30a, as determined by Western blot analysis (Fig. 1A). Down-regulation of SRp30a induced an increase in the caspase-9b splice variant at the expense of caspase-9a, thereby
inducing a decrease in the caspase-9a/9b ratio from 1.67 ± 0.11 to 0.56 ± 0.08 (* P < 0.005) (Fig. 1B), which translated to the protein level (20). To demonstrate the specificity of the SRp30a siRNA and control for off-target effects, we also examined individual siRNAs (SRp30a-1, SRp30a-2, SRp30a-3, and SRp30a-4) against SRp30a. Each individual siRNA to SRp30a induced an increase in the caspase-9b splice variant and a concomitant decrease in the caspase-9a splice variant, demonstrating that the effect on caspase-9 pre-mRNA processing is specific for the downregulation of SRp30a and not the result of off-target effects of the siRNA (data not shown). Downregulation of the other splicing factors (confirmed by Western immunoblotting as >75%) listed in Table 1 by RNAi technology did not induce any change in the pre-mRNA processing of caspase-9.

The effect of the downregulation of SRp30a on caspase-9 pre-mRNA processing was not attributable to a generalized effect on the RNA splicing machinery, as the alternative splicing of Bcl-x and Bax was unaffected (Fig. 1C). Therefore, these data demonstrate that SRp30a specifically regulates the alternative splicing of caspase-9, and this effect on the caspase-9a/9b ratio is not attributable to an effect on the overall activity of pre-mRNA processing.

**SRp30a is required for ceramide to affect the alternative splicing of caspase-9 pre-mRNA**

Exogenous ceramide was previously shown to regulate the alternative splicing of caspase-9 (20). Based on these data, we hypothesized that downregulation of the critical RNA trans-acting factor would inhibit the ability of ceramide to modulate the alternative splicing pattern of caspase-9 pre-mRNA. Furthermore, we hypothesized that if ceramide modulates the alternative splicing of caspase-9 via a different RNA trans-factor, then an activation of caspase-9a pre-mRNA processing will overcome the induction of caspase-9b pre-mRNA processing by SRp30a siRNA.

To determine this, A549 cells subjected to SRp30a or control siRNA transfection were treated with 20 μM d-erythro-C6 ceramide for 12 h (the minimum time required for exogenous ceramide to affect the alternative splicing of caspase-9). Analysis of the alternative splice variants of caspase-9 revealed that ceramide treatment in the absence of SRp30a siRNA activated the caspase-9a alternative splicing, as reported previously (20), changing the caspase-9a/9b ratio from 1.66 ± 0.22 to 3.29 ± 0.22. A549 cells treated with only SRp30a siRNA also affected the alternative splicing of caspase-9.

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**TABLE 1. A complete list of splicing factors analyzed by RNAi technology for involvement in the alternative splicing mechanism of caspase-9 pre-mRNA**

| SR Family Proteins | Suggested by the Literature |
|--------------------|-----------------------------|
| SRp30a*            | hnRNP A1α, GAS11            |
| SRp30c             | hnRNP B1, PUF60             |
| SRp40              | SAM68, PTB1                 |
| SRp46              | SAP155, PTB2                |
| SRp30b             | RAB11                       |

RNAi, RNA interference. Proteins were chosen for their possible relevance to the regulation of alternative splicing of caspase-9 pre-mRNA as suggested by previously published findings from our laboratory (SR proteins) as well as other findings in the literature (15, 20, 26, 35).

*Splicing factor that may affect the selection of alternative splicing sites of caspase-9, as determined by RNAi technology and examination of caspase-9 splice variants by RT-PCR assay.
After 36 h of incubation, cells were treated with 20 μM D-e-C₆ ceramide, resulting in a caspase-9a/9b ratio of 0.32. This significantly affects the alternative splicing of caspase-9. Cells subjected to SRp30a siRNA transfection did not experience this decrease in the antiapoptotic splice variant caspase-9a, with a concomitant increase in the apoptotic splice variant caspase-9b (0.22 to 0.43). Ceramide treatment of MCF-7 cells significantly affects the sensitivity of these cells to undergo apoptosis in response to a variety of apoptotic stimuli (37).

Previously, our laboratory reported that ceramide induces, via alternative splicing, the expression of the pro-apoptotic splice variant caspase-9a, with a concomitant decrease in the antiapoptotic splice variant caspase-9b (20). In this study, the SR protein, SRp30a, has been identified as an RNA trans-acting factor that functions in the regulation of the alternative splicing of caspase-9 pre-mRNA. These findings are important for several reasons. First, they provide the first report of mechanistic insight for the regulation of the alternative splicing of caspase-9. Second, they provide further insight into the ceramide-mediated mechanism for the regulation of the apoptotic signaling pathway. Finally, they illustrate a potential drug target for anticancer therapies.

Many reports in the literature document the roles of caspase-9a and its dominant-negative splice variant, caspase-9b, in regulating the apoptotic signaling pathway at the level of apoptotic protease activating factor-1 (Apaf-1) complex formation (34–38). Furthermore, studies indicate that tight regulation of the splicing mechanism of caspase-9 is critical for proper cellular signaling, as manipulation of the caspase-9a/9b expression ratio in MCF-7 cells can significantly affect the sensitivity of these cells to undergo apoptosis in response to a variety of apoptotic stimuli (37).

Despite this importance in the regulation of the expression of the caspase-9 splice variants (caspase-9a/9b ratio), to date, there is no understanding of the mechanism of the alternative splicing of caspase-9 pre-mRNA. These studies begin to elucidate the critical splicing components involved in this alternative splicing mechanism. We demonstrated that downregulation of SRp30a using RNAi technology dramatically shifted the caspase-9a/9b ratio by inducing caspase-9b mRNA levels at the expense of caspase-9a. The downregulation of SRp30a had no effect on the alternative splicing of Bcl-x and Bax, suggesting that the pre-mRNA processing of caspase-9 is distinct from these apoptotic factors, indicating a novel mechanism of alternative splicing mechanisms for the regulation of caspase gene expression. Thus, SRp30a, a well-established regulator of alternative splicing mechanisms, plays a critical role in the alternative splicing mechanism of caspase-9 pre-mRNA. Furthermore, unpublished findings from our laboratory, sequence analysis of the caspase-9 gene revealed the identification of purine-rich exonic splicing enhancers, sequences commonly associated with SRp30a, located in the peripheral exons of the exon 3, 4, 5, and 6 cassette. These findings are consistent with the involvement of SRp30a in the alternative splicing mechanism of caspase-9 and implicate binding regions for the SRp30a interaction with the caspase-9 pre-mRNA transcript at the external exons. Together, these data are the first to establish a critical splicing factor in the alternative splicing mechanism of caspase-9 pre-mRNA.

This study also sheds light on the mechanism by which ceramide affects the alternative splicing of caspase-9 pre-mRNA. This is based on the finding that SRp30a induces caspase-9b alternative splicing and blocks the ability of ceramide to induce caspase-9a pre-mRNA processing, as demonstrated previously (20). Previous findings from our laboratory demonstrated that de novo ceramide generation and PP1 activation induced the dephosphorylation of SR proteins and caspase-9a pre-mRNA processing (20), and findings from this study demonstrated the ability of SRp30a downregulation to block ceramide activation of the exon 3, 4, 5, and 6 cassette inclusion mechanism of
caspase-9a pre-mRNA processing. These studies now establish a direct link between the generation of de novo ceramide, SRp30a, and the regulation of caspase-9 pre-mRNA processing and suggest a possible role for PP1 and SRp30a (de)phosphorylation (Fig. 3). The participation of PP1 is consistent with findings by Cardinali, Cohen, and Lamond (39) that the dephosphorylation of SR proteins by PP1 induces the activation of the cryptic splice variant of adenopre-mRNA. Furthermore, studies by Xiao and Manley (40) have demonstrated a phosphorylation-dependent mechanism for SRp30a interaction with specific RNA cis elements and additional splicing factors for splice site activation. However, further studies are warranted to determine the role of PP1 activation by ceramide in the dephosphorylation of SRp30a and consequent effects on the alternative splicing pattern of caspase-9 pre-mRNA.

Many reports in the literature demonstrate that the physiological outcome of a cell can be determined by the proportion of antiapoptotic and proapoptotic factors (34, 35). Thus, the antagonistic functions of the alternative splice variants of the caspase-9 gene in apoptosis may provide an additional method of regulating cell fate. One possible mechanism by which the caspase-9 splice variants regulate apoptosis is via proper assembly of the apoptosome. In this respect, studies have shown that the interaction of Apaf-1 and procaspase-9a is a critical interaction in the apoptotic signaling pathway for inducing cell death, and interference with this interaction can hinder the induction of apoptosis (34–38). The dominant-negative splice variant of caspase-9a, caspase-9b, is one source of such interference (37, 41). This was demonstrated by Alnemri and coworkers (37), who showed by in vitro binding assays that the short isoform of caspase-9, caspase-9b, can function as a competitive inhibitor that equally interacts with Apaf-1 to prevent the formation of a functional apoptosome, thereby blocking subsequent procaspase-9a and caspase-3 activation. Because the interaction of Apaf-1 and procaspase-9a is the focus of most apoptotic signals, interference with this complex formation can contribute to tumor development and progression. Thus, the alternative splice variant, caspase-9b, can negatively regulate apoptosis by a dominant-negative mechanism. In this regard, unpublished findings from our laboratory demonstrated that the caspase-9a/9b ratio is significantly decreased in transformed A549 cells compared with nontransformed bronchial epithelial cells. Therefore, dysregulation of caspase-9 pre-mRNA processing may contribute to the proliferation of cancer cells and cellular transformation by oncogenes, suggesting a role for the alternative splicing mechanism of caspase-9 as a potential drug target for anticancer therapies.

In conclusion, these results demonstrate the identification of an RNA trans-acting factor that functions to regulate the pre-mRNA processing of caspase-9, thereby providing initial insight into the caspase-9 alternative splicing mechanism. These data establish a direct link between ceramide, SR proteins, and the alternative splicing of an apoptotic factor, demonstrating a novel mechanism for the ceramide regulation of apoptotic signaling. Because the ratio of caspase-9 splice variants (caspase-9a/9b) was shown to have direct relevance in the sensitization of cells to a wide variety of apoptotic agents, this mechanism may have significance in drug resistance and chemotherapeutic sensitivity and, thus, may provide a new drug target for anticancer therapies.

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