Chemical Biology of Splicing Inhibitors

スプライシング阻害剤に関する化学生物学的研究

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Abstract:

Splicing of primary transcripts (pre-mRNA) removes non-coding intronic sequences and joins exonic sequences to make a mature mRNA that can be transported from the nucleus into the cytoplasm and translated into protein. Thus, splicing is a key mechanism for regular gene expression and interference with splicing will affect gene expression. Deregulation or inhibition of splicing will result in production of non-functional or even deleterious proteins. Recently, FR901464 and its methylated derivative Spliceostatin A (SSA) were reported for the first time to inhibit pre-mRNA splicing by binding non-covalently to the SF3b sub-complex in the U2 snRNP. SSA is chemically more stable than its parent molecule FR901464, which was originally isolated from a fermentation broth of *Pseudomonas sp*. During screening of microbial metabolites, FR901464 was found to activate viral promoters including those of the SV40 and the cytomegalovirus (CMV). Previous studies have shown that SSA leads to transcriptional repression of about 20% of expressed genes, while enhancing transcription of only a small subset. Among the most highly up-regulated genes was Interleukin 8 (IL-8). Since IL-8 and CMV promoters are activated by a similar set of transcription factors, I used luciferase reporter constructs of each promoter for a comparative study.

IL-8 is a well-studied CXC family chemokine that activates and recruits leukocytes to the site of inflammation. Interleukin 1 (IL-1), tumor necrosis factor alpha (TNF-α) and phorbol 12-myristate 13-acetate (PMA) are well known inducers of IL-8. These inducers can enhance the transcriptional stimulatory activity of viral promoters (SV40 and CMV) as well and are also able to activate cellular signaling kinases. Previous investigations suggested

that IL-8 production is regulated by extracellular signal-regulated protein kinase (ERK) and transcription factor NF-kB in response to TNFα, IL-1β or PMA. To better understand the underlying mechanism we investigated both possible upstream signaling pathways as well as likely transcription factors responsible for IL-8 induction. The mitogen-activated protein kinases (MAPKs) are important signaling kinases that activate a variety of transcription factors through phosphorylation. Here I report that SSA treatment induces ERK activation and that SSA treatment leads to enhancement of NF-kB activity.

Acknowledgment

I offer my enduring gratitude to the faculty, staff and my fellow students at the RIKEN and Saitama University, who have inspired me to continue my work in this field. I owe particular thanks to Dr. Minoru Yoshida, whose penetrating questions taught me to question more deeply.

I thank Dr.Tilman Schneider-Poetsch for enlarging my vision of science and providing coherent answers to my endless questions. I would just describe him in one word “Brilliant”.

Special thanks are owed to my parents; whose have supported me throughout my years of education, both morally and financially.

Abbreviations

Ac-SSA	Acetylated SSA
AP-1	Activated protein 1
ASK1	Apoptosis regulating kinase 1
ATF6	Activating transcription factor 6
BPS	Branch point sequence
b-SSA	Biotinylated SSA
C/EBP	CCAAT/enhancer-biding protein
CDK	Cyclin-dependent protein kinase
CMV	Cytomegalovirus
COX-2	Cyclo oxygenase 2
CREB	cAMP response element-binding protein
DMSO	Dimethylsulfoxide
EDTA	Ethylene diamine tetraacetic acid
EGTA	Ethylene Glycol tetraacetic acid
ER	Endoplasmic reticulum
ERAD	ER-associated protein degradation
ERK	Exracellular signal-regulated protein kinase
FR	FR901464
GDP	Guanosine diphosphate
IL-6	Interleukin 6
IL-8	Interleuikin-8
IκBα	I kappa beta alpha
iNOS	Inducible nitric oxide synthase

IRE1	Inositol-requiring Enzyme 1
JNK	JUN N-terminal kinase
LC-MS/MS	Liquid chromatography-t andem mass spectrometry
MAPK	Mitogen-activated protein kinase
MeOH	Methanol
MnSOD	Manganese Superoxide dismutase
mRNA	messenger RNA
NF-κB	Nuclear factor kappa Beta
NOD-1	Nucleotide-binding oligomerization domain-containg protein 1
p65	65 KD component of NF-kB
p300/CBP	EP300 or EIA binding protein/CREB-binding protein
Parth	Parthenolide
PCAF	p300/CBP-associated factor
PCR	Polymerase chain reaction
PIC	Protease inhibitor cocktail
PDI	Protein disluphide isomerase
PERK	Pancreatic ER kinase
Plad B	Pladienolide B
PMA	Phorbol 12-myristate 13-acetate
PMSF	Phenyl methane sulfonyl fluoride
PPT	Polypyrimidine tract
qPCR	Quantitative Polymerase Chain Reaction

SAP	Spliceosome-Associated protein
SF1	Splicing factor 1
SF3b	Splicing factor 3b
SiRNA	Small interference RNA
SnRNP	Small ribonucleoprotein
SSA	Spliceostatin A
SV40	Simian viruss 40
SOS	Son of sevenless
SOD-2	Superoxide dismutase 2
Tg	Thapsigargin
TMX	Thioredoxin-related transmembrane protein
TNFAIP3	Tumor necrosis factor, alpha induced protein 3
TNF-α	Tumor necrosis factor alpha
U2AF	U2 auxiliary factor
UPR	Unfolded protein response
VCAM-1	Vascular cell adhesion molecule 1
VEGF	Vascular endothelial growth factor VCAM-1
XBP1	X-box binding protein 1
XBP1-s	X-box binding protein 1 spliced
XBP1-u	X-box binding protein 1 unspliced

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Chapter 1

Introduction

1.1 What is splicing?

A hallmark of eukaryotic gene regulation lies in pre-mRNA splicing, a process in which intronic sequences are removed from a primary transcript to form a mature mRNA message. I will provide a brief overview of the RNA splicing mechanism and the proteins and ribonuclear complexes involved in splicing.

In eukaryotes pre-mRNA is processed with high precision and control inside the nucleus. The pre-mRNA is subjected to 5′-end capping, 3′-end cleavage and polyadenylation to become a mature mRNA, which is then transported into the cytoplasm for translation (1). Splicing can be direct that involved cis-acting sequences in pre-mRNA and alternate splicing that is regulated by binding of trans-acting proteins to cis-acting sequences on the primary transcript itself. Alternate splicing includes or excludes particular exons of a gene from the final processed mRNA. This process leads to the production of multiple mRNA from a single gene. Thus, alternate splicing increase protein diversity and each isoform have different biological function (2).

A complex of proteins called the spliceosome carried out direct splicing. The spliceosome contains five major complexes composed of RNA and proteins called small ribonucleoproteins (snRNPs) and 100-150 other small proteins. The five major snRNPs are abbreviated U1, U2, U4, U5 and U6. The cooperative action of all these complexes with accessory factors excises the introns from pre-mRNA. Each snRNP has its own target site for binding on pre-mRNA (3, 4).

A typical intron is defined by its 3’ and 5’ splice sites (3’ss and 5’ss). Approximately 18-40 nucleotide upstream of the 3′ splice site lies the branch point sequence (BPS), while the, polypyrimidine tract (PPT) is positioned between BPS and the 3′ss. In splicing the U1 snRNP first binds the 5′ splice site. Base pairing between 5′ss and U1 snRNP are important for the initiation

of the splicing process. Next splicing factor 1 (SF1) binds the BPS followed by the recruitment of the U2 accessory factor (U2AF), consisting of two subunits (U2AF65 and U2AF35) to the PPT and 3′ss respectively. This assembly is named E complex. In the next step U2 snRNP is recruited to the BPS and replaces SF1, which results in the formation of A complex. Next is the formation of B complex by recruitment of the U4/U6-U5 tri-snRNP complex. The last step is achieved by C complex in which U1 and U4 snRNPs dissociate resulting in the catalytically active spliceosome. Two sequential transesterification reactions occur in active complex C. The first transesterification reaction involves the nucleophilic attack of the 2′ hydroxyl group of adenosine in branch point sequence on the 5′ splice junction. This reaction produces a lariat structure. The second transesterification reaction involves the nucleophilic attack of the released 3′ hydroxyl group of the 5′ exon on the 3′ splice junction. The end result is removal of intronic sequence and forming of phosphodiester bond between the 5′ and 3′ exons. The overall schematic diagram of SF3b binding and spicing mechanism is shown in (Fig. 1.1) and (Fig. 1.2) (5).

1.2 Discovery of SSA and its derivatives

In 1996, the Fujisawa Pharmaceutical Co. isolated three structurally related compounds from a fermentation broth of the bacterium *Pseudomonas sp. No. 2663* designated FR901463, FR901464, and FR901465 during a screen for microbial metabolites that enhance the transcriptional regulation of the SV40 promoter (4). The basic hypothesis was to screen for transcription factor regulators that might be a new class of antitumor agent. All three were found to enhance the transcriptional activity of the SV40 promoter and also exhibited potent antitumor activity against different human solid tumor cell lines. FR901464 proved the most potent and also showed efficacy in extending the lifespan of tumor bearing mice (6, 7). FR901464 causes cell cycle arrest at G1 and G2/M phases and induces DNA fragmentation (8). In 2007, Yoshida and co-workers reported a more chemically stable derivative of FR901464 with similar antitumor activity, named Spliceostatin A (SSA). Furthermore, they also found that SSA inhibited splicing *in vitro* and led to accumulation of pre-

mRNA (9). Currently available derivatives of SSA are biotinylated probe called as biotin-SSA, used for isolation of target proteins by affinity pulldown. This derivative was used to specifically isolate the SF3b subcomplex of the U2 snRNP (9). Another derivative called Ac-SSA is actually SSA acetylated on the C4 hyrdroxyl group proved inactive (8). An Ac-SSA was used in further experiments as negative control.

Spliceostatin A was the first splicing inhibitor reported but currently several splicing inhibitors have been identified, including pladienolide B (Pla B), which acts via a similar, if not identical mechanism to SSA. The structures of FR901464, SSA, Ac-SSA and pladienolide B are shown in table 1.1.

1.3 Other molecules targeting pre-mRNA splicing

An antitumor macrolide, pladienolide, isolated from a fermentation broth of Streptomyces platenensis, displayed inhibitory activity against expression of vascular endothelial growth factor (VEGF) (10). Pladienolide proved to inhibit splicing as well. Crosslinking studies with biotinylated and photo affinity tag-equipped pladienolide B identified the SAP130 subunit of SF3b as its likely target (11). While having vastly different chemical structures SSA and pladienolide B seem to have a nearly identical effect on splicing. As structural information on the spliceosome remains limited and as their exact binding sites still await identification, it remains unclear whether they indeed bind to the same site of the SF3b subcomplex. (12). Both were found to inhibit splicing and cause the nuclear retention of unspliced RNA (Fig. 1.3). However, several unspliced pre-mRNAs appear to leak into the cytoplasm and getting translated. The best-studied case is p27*, the product of unspliced mRNA of the p27 cyclin-dependent kinase (CDK) inhibitor. The p27 is degraded by the 26S proteasome while the C-terminal truncated $p27^{*}$ exhibit resistivity to proteasome inhibitor MG132. The resistance to proteosomal degradation is because of the lack of the C-terminal phosphoryaltion site (T187) in $p27^{*}$, which is subjected to ubiquitination and subsequent proteosomal degradation. The persistent existence of $p27^{*}$ might be responsible for arresting the cell cycle in cancer cells (9).

In the course of the past years further splicing inhibitors have been identified. In 2008, O'Brien K et al, isolated isoginkgetin from the plant Ginkgo biloba. The mode of action of isoginkgetin is different from SSA and pladienolide. Isoginkgetin inhibits the recruitment of the U4/U5/U6 tri-snRNP, thus preventing B complex formation and resulting in accumulated A complex. The exact target of isoginkgetin in the spliceosome is unclear. Several other spicing inhibitors were isolated from different sources, with different structures and modes of action, for example TG003 that targets Clk1 and Clk4 (13), anacardic acid and garcinol targets the inhibition of p300/CBP and Gcn5/PCAF splicing at A complex (14, 15).

1.4 FR901464 induces ER stress

Besides its effect on splicing, Tashiro et al. identified an additional FR901464-binding protein using biotinylated SSA and LC-MS/MS. They identified TMX (Thioredoxin-related Transmembrane Protein) as another FR901464 binding protein (unpublished data). In contrast to the spliceosomal proteins, the interaction between FR901464 and TMX appears to be covalent via Cys-56 in the thioredoxin-like domain of TMX. TMX localizes to the Endoplasmic Reticulum and plays an important role in the unfolded protein response (UPR). The role of UPR is to maintain normal ER function under stress conditions by enhancing the folding capacity of the ER and promoting ER-associated protein degradation (ERAD) to dispose off unfolded and misfolded proteins (16). When the amount of aggregates exceeds the folding capacity of the ER, the condition is known as ER stress. Protein aggregation is toxic to cells; consequently, ER stress activates a complex signaling network to reduce the burden on the ER. While FR901464 treatment induces ER stress, it failed to do so when TMX was knocked down. Therefore it appeared that FR901464-induced ER stress depends on the molecule's interaction with TMX and could potentially be independent on its effect on splicing.

1.5 SSA-induced gene activation

To better understand the underlying mechanism, I investigated both possible upstream signaling pathways as well as the likely transcription factors responsible. I found that SSA treatment led to increased ERK kinase activity and that chemical inhibition of ERK also lessened activation of the cytomegalovirus (CMV) or Interleukin-8 (IL-8) promoters. Systematic deletion studies of transcription factor binding sites of the IL-8 promoter suggested that NF-kB activation is mainly responsible for SSA-induced expression of CMV and IL-8 promoters.

Tunicamycin and thapsigargin are well-known inducers of ER stress. It has been reported that ER stress inducers can activate the NF-kB pathway. I further found that thapsigargin induced IL-8 promoter activity and up-regulated NF-kB target genes. In order to check the link between ER stress and NF-kB activity to bring about the expression of CMV and IL-8, I checked IL-8 promoter activity in TMX knockdown cells upon SSA treatment. SSA was found to enhance IL-8 promoter activities to similar extent in control siRNA and TMX-knockdown cells, clearly suggesting that SSA–induced gene activation is independent of ER stress.

1.5 Aim of this study

The chemical genetics laboratory at RIKEN aims to identify novel bioactive natural compounds that modulate regulation of protein post-translational modifications and their development into biological probes. Considering its dramatic effect, not only on mRNA metabolism but also gene regulation in general, as well as its antitumor activity, a further investigation into SSA's mechanism appeared prudent. It is estimated that 15% of all mutations that cause human genetic diseases, such as autoimmune disorders, neurodegenerative diseases, cystic fibrosis, growth hormone deficiency, muscular dystrophy and cancer are associated with aberrant splicing. Thus chemical inhibition of splicing carries potential for drug development. In clinical studies splicing inhibitors showed limited efficacy but also significant side effects. As the screening and development of pre-mRNA processing modulators has just begun, screening of further small molecules or derivatives of SSA with fewer side effects has become an active field of investigation. Identification of particularly susceptible tumors may also increase the clinical utility of splicing inhibitors. As the spliceosome remains incompletely understood, further chemical modulators would prove immensely useful in gaining a better appreciation for RNA-mediated RNA processing.

With its clinical potential in mind, gaining a better understanding of SSA's mechanism and cellular effect remains worthwhile. In the current study I focused on interleukin-8 (IL-8) and the cytomegalovirus (CMV) promoters as a model to investigate the effect of SSA on gene expression. Both IL-8 and CMV promoters recruit a similar subset of transcription factors. Here I report that SSA treatment induces ERK activation and leads to enhance NF-kB activity. In direct comparison to SSA, pladienolide B's effect remained indistinguishable.

Figures Chapter 1:

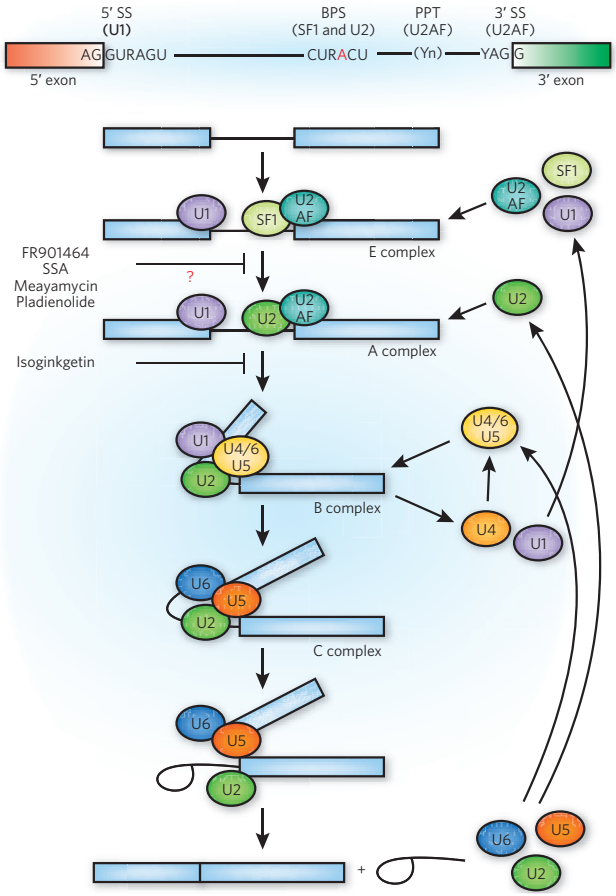
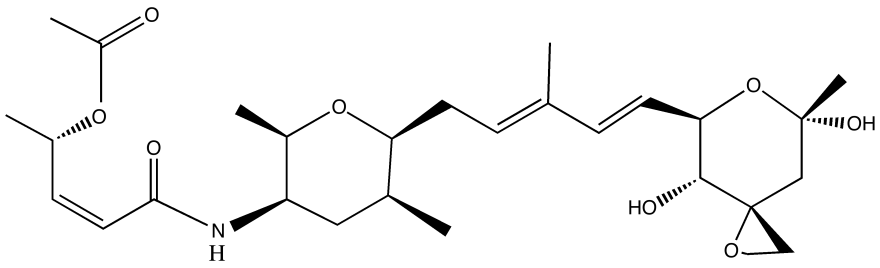
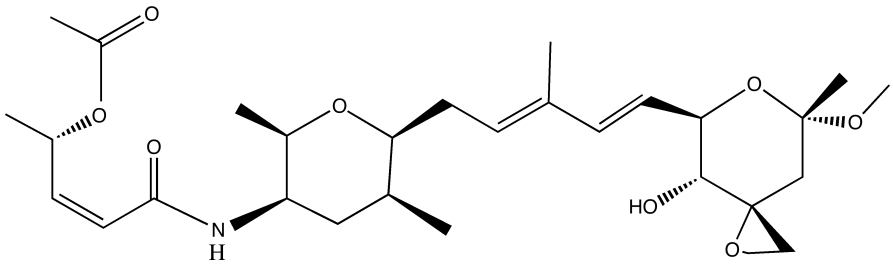


Figure 1.1: Overview of the splicing process, highlighting the steps inhibited by small molecules. Splicing is initiated by recognition of the 5′ splice sites by the U1 snRNP, followed by 3′ splice site recognition by heterodimeric U2 snRNP auxiliary factor (U2AF), which leads to the formation of E (early) complex. Then U2 snRNP is recruited to the branch point sequence (BPS) in an ATP-dependent manner to form A complex. Next the U4-U6-U5 tri-snRNP is recruited to form B complex, which is resolved into the catalytically active C complex with the release of U1 and U4.

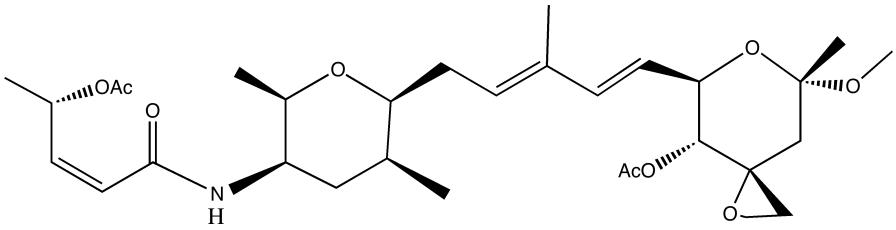
Table 1:



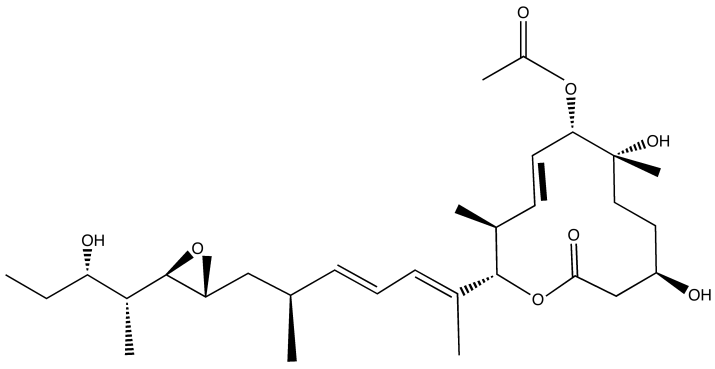
FR901464



Spliceostatin A

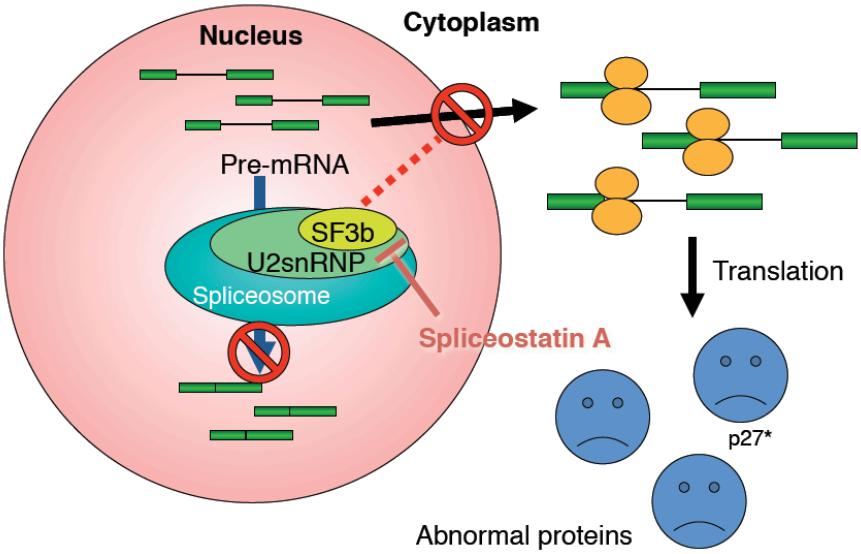


Acetylated spliceostatin A



Pladienolide B

Table 1.1: Structure of splicing inhibitors and their derivatives:



1.2: SSA inhibits splicing by binding to SF3b and retains pre-mRNA in the nucleus: Binding of SSA to SF3b impairs splicing. A subset of pre-mRNA leak into the cytoplasm, where they may be translated into likely truncated and not necessarily functional proteins.One of the known protein is p27* and it might contribute to the cell cycle arrest.

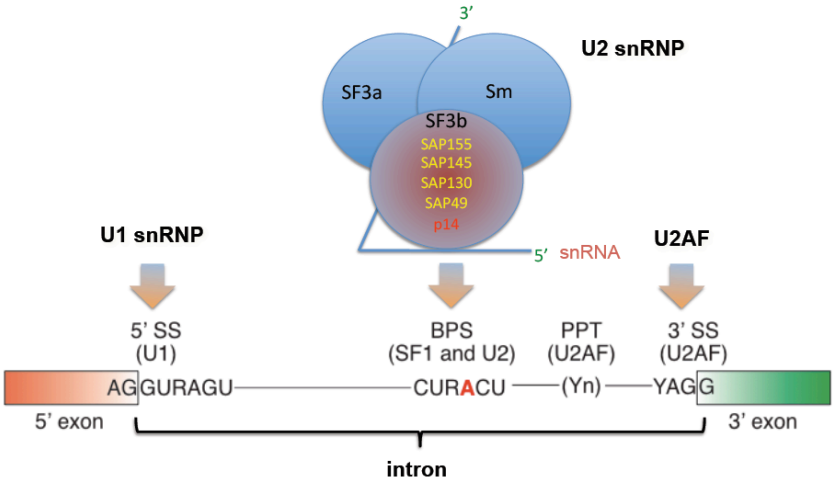


Figure 1.3: SF3b recognizing the branch point sequence within the intron of pre-mRNA. A typical U2 snRNP consist of SF3a, Sm proteins and SF3b. SF3b contain five different proteins, abbreviated as SAP130, SAP14, SAP155, SAP149 and p14. The sub-component of SF3b, p14 interacts directly with the pre-mRNA branch adenosine.

Chapter 2:

SSA enhances the transcriptional activation of IL-8 and CMV promoters.

2.1: introduction

As the search for more potent anti-tumor agents continues, various strategies are being pursued, many involving high throughput screening of large libraries of artificial or natural compounds. Almost two decades ago, Nakajima et al, used an approach that utilized SV40 promoter transcriptional regulation by compounds with unknown targets in hope to develop a new anti tumor agent with a novel mode of action. Transcriptional regulation controls different events in tumor cell growth such as cell cycle transition, cell death and production of growth factors etc. The basic hypothesis was to search for transcriptional up-regulators of the SV40 promoter, as it was believed that a compound activating transcription might increase the production of cancer suppressor gene products that will eventually help in combating cancer.

During their screening of microbial metabolites, FR901464 was found to enhance the transcriptional regulation of SV40. It displayed potent antitumor activity arresting the cell cycle at G1 and G2/M phases and down regulating several endogenous genes. Studies over the past decade showed that SSA dependent suppression of gene expression is not only due to splicing inhibition but also through reduced RNA Pol II recruitment, for example in case of the VEGF (vascular endothelial growth factor) gene.

Although a sizeable number of genes are down-regulated, interestingly, few tumor-related genes are up-regulated under SSA treatment. Keeping in view the anti tumor activity of SSA, it seems important to elucidate the molecular mechanism by which SSA up-regulates these genes. In the current study I focused on the interleukin-8 (IL-8) and the strong viral promoter of the cytomegalovirus (CMV) as a model for investigating the effect of SSA on gene

expression. Both promoters were linked to a luciferase reporter gene and SSA-induced gene activation was quantified by using three approaches, namely (1) qPCR, (2) luciferase assay and (3) western blot. I assumed that SSA would stimulate both transgenes in a similar manner as both IL-8 and CMV promoters recruit a similar subset of transcription factors. Our data confirmed that the stimulation is time and dose dependent. Furthermore, pladienolide B also enhanced transcriptional activation while Ac-SSA fails to do so. On the other hand, knockdown of components of SF3b (SAP130, SAP145 and SAP155) also increased the production of luciferase.

2.2 Materials and Methods

2.2.1 Materials and cell lines

All compounds were prepared asstock solutions in methanol for SSA and Ac-SSA, while pladienolide B was prepared in DMSO. All stocks were maintained at -20 °C. NIH3T3, NCL-5 and Hela cells were maintained in DMEM with 10% FBS. The NCL-5 cell line is basically a NIH3T3 cell line that is stably expressing luciferase under the control of the CMV promoter (Young Bae Kim et al, Oncogene. 1999).

2.2.2 Antibodies and Immunoblotting

Antibodies against luciferase (sc-32896), SAP145 (sc-101133), were purchased from Santacruz Biotechnology. Antibodies against SAP 130, SAP155 and β-actin were purchased from (Abcam). Mouse monoclonal anti α-Tubulin (B-5-1-2) antibody was from Sigma. Cells were lysed in NETN lysis buffer (50mM tris-Hcl pH7.5, 150mM NaCl, 5mM EDTA, 0.1% Nonidet P-40), containing 1% protease inhibitor Coktail (Roche) and protein concentrations were determined. Cell lysate was dissolved in 1XSDS–PAGE sample buffer and heated at 95 °C for 5 min. 20 μg of total protein were resolved on 10% SDS–PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore) by electroblottting. Membranes were blocked in skim milk followed by incubation with primary and secondary antibodies and immune complexes detected by Immobilon™ Western Chemiluminescent HRP substrate (Millipore) in a LAS-3000 image analyzer (GE Healthcare).

2.2.3 qPCR analysis

Total RNA was prepared from drug-treated cells with the RNeasy Mini Kit (QIAGEN) and cDNA was prepared by reverse transcription with random 9-mer primers using the RNA PCR Kit (AMV) Ver.3.0 (Takara Bio Inc., Shiga, Japan). Primers for target genes were designed by the Universal Probe Library Assay Design Centre (Roche Applied Science) and gene expression was analyzed using the qPCR SYBR Premix Ex Taq II (Takara BioInc.) on a

LightCycler 480 (Roche).

2.2.4 Expression and knockdown

NCL-5 and NIH3T3 cells were seeded at $5x10^4$ cells per well in a 24 well multi-well plate and transfected with expression vectors using the Lipofectamine™ LTX reagent (Invitrogen) for 48 h and FuGENE® HD reagent (Roche) for 72 h. The pCR3.1-Luc representing wild type CMV promoter was kindly provided by Dr. Georg F. Weber (He B et al, BBRC. 2004). The construct containing the IL-8 promoter linked to luciferase was kindly provided by Dr. Naofumi Mukaida (Naoki Mori et al, Cancer Research. 1988). Cells were lysed in NETN buffer after treatment with drug for the indicated time points, luciferase activity was measured using a SpectraMax L (Molecular Devices). Small Interferening RNA (siRNA) for SAP-130, SAP-145, SAP-155 and control non-specific siRNA sequences from Dharmacon was introduced according to the manufacturer's instructions.

2.3 Results

2.3.1 Effect of SSA on IL-8 and CMV promoter activity

Previous studies suggested that SSA enhances the transcription regulation of viral promoter SV40 in M-8 cells and microarray data of HeLa cells treated with SSA showed a significant production of IL-8 (7, 17). As SV40 represents an artificial construct, it may not represent physiologically relevant conditions faithfully enough. Therefore, I decided to check transgene activation endogenously and exogenously, using an IL-8 as an endogenous gene, sensitive to SSA treatment. The cellular mRNA levels were monitored, while also luciferase reporter constructs were transfected for easier readout.

For reproducibility and confirmatory of previous findings, western blot, luciferase assay and qPCR were performed to assess the expression of the IL-8 gene and another strong viral promoter, the CMV promoter in response to SSA as shown in (Fig. 2.1 A-F). Our results confirmed SSA-induced gene activation at exogenous and endogenous level.

The endogenous level of IL-8 expression gene was quantified by qPCR. HeLa cells endogenously expressing IL-8 were used and primers that specifically recognize exon-exon junctions were designed. Furthermore, we also checked IL-8 gene activation in A549 cells (Data not shown). For luciferase assays, NIH3T3 cells were transiently transfected with IL-8 promoter containing constructs for 48 h, followed by SSA treatment for 12 h. Cells were collected in NETN lysis buffer, sonicated and protein concentration was determined by Bradford assay. Equal amounts (20 µg) were utilized for luciferase assay and western blot. Anti-luciferase antibody was used for quantifying IL-8 promoter activity at protein level and β-actin was used as a loading control. These results clearly suggest that SSA induces gene expression exogenous and endogenous level.

I further used NCL-5 cells that stably express a luciferase reporter gene under the control of the CMV promoter. Results (Fig. 2.1 D-E) suggest that

SSA enhances the transcriptional activation of the viral promoter at mRNA and protein level.

2.3.2 Transcriptional activation in a time and dose dependent manner by SSA

Transcriptional activation of the CMV promoter was time and dose dependent. I confirmed the time dependent activation of the CMV promoter by keeping a constant concentration of SSA (100 ng/ml) and varying the time of incubation with SSA. SSA was added to NCL-5 cells at 1, 3, 6 and 12 h, followed by qPCR for quantifying the mRNA level of the luciferase gene shown in (Fig. 2.2 A). Time dependent activation of the CMV promoter was confirmed at protein level by incubating 100 ng/ml SSA at 2, 4, 7 and 24 h, followed by luciferase assay as shown in Fig 2.2 B. The next approach utilized different concentration SSA (5, 20 and 100 ng/ml) at 12 h as shown in (Fig. 2.3 A). Furthermore, pladienolide B showed similar a effect while the inactive form of SSA (Ac-SSA) failed in doing so. The maximum increase of CMV promoter activity observed was about 16 fold over methanol 100 ng/ml SSA concentration. I checked the activity of CMV promoter at 500 ng/ml SSA treatment but did not notice any significant difference between 100 ng/ml and 500 ng/ml SSA treatment (data not shown). These results showed that CMV promoter induction is initiated after 2 or 3 h and persistently increases with time and dose.

2.3.3 Effect of splicing inhibitors and splicing inhibition on gene activation

In order to investigate the role of splicing inhibition upon the activation of IL-8 and CMV, I used paldienolide B and Ac-SSA. As I can clearly see in (Fig. 2.1 A) that vehicle (methanol) and Ac-SSA failed to increase the level of IL-8 mRNA. We also checked CMV and IL-8 promoter stimulation in response to pladienolide B and Ac-SSA (Fig. 2.3 A-B). Furthermore, we knocked down components of SF3b subunits abbreviated as SAP. Individual knockdown of SAP proteins (SAP130, SAP145, SAP155) were able to induce the CMV

promoter as shown in (Fig. 2.3 C). The existing data suggest gene activation is sensitive to splicing inhibitors and splicing inhibition.

2.4 Conclusion:

Here I demonstrated that SSA activates IL-8 and CMV promoters at mRNA and protein levels. As, SSA and pladienolide B share the same target; I checked the CMV promoter induction by addition of SSA and pladienolide B together. I could not find any significant difference in terms of increase, which suggesst that SSA and pladienolide B might affect a similar pathway for IL-8 and CMV promoter induction. Furthermore, gene activation was observed in SAP knockdown cells further suggesting that induction of CMV and IL-8 genes are splicing inhibition dependent. I concluded that transgene activation is dose and time dependent manner upon splicing inhibition.

Figures chapter 2:

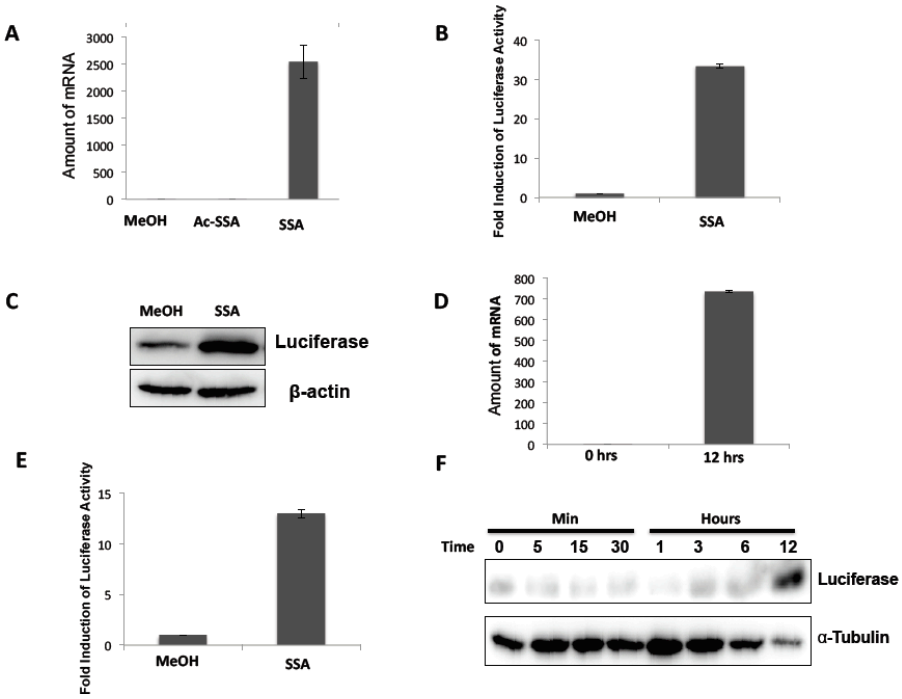


Figure 2.1. Spliceostatin A (SSA) treatment leads to CMV and IL-8 promoter activation.

(A) SSA induced expression of the IL-8 promoter, measured by qPCR, (B) luciferase reporter and relative concentration of the luciferase protein by (C) Western Blot. Similar results were observed with the CMV promoter (D-F).

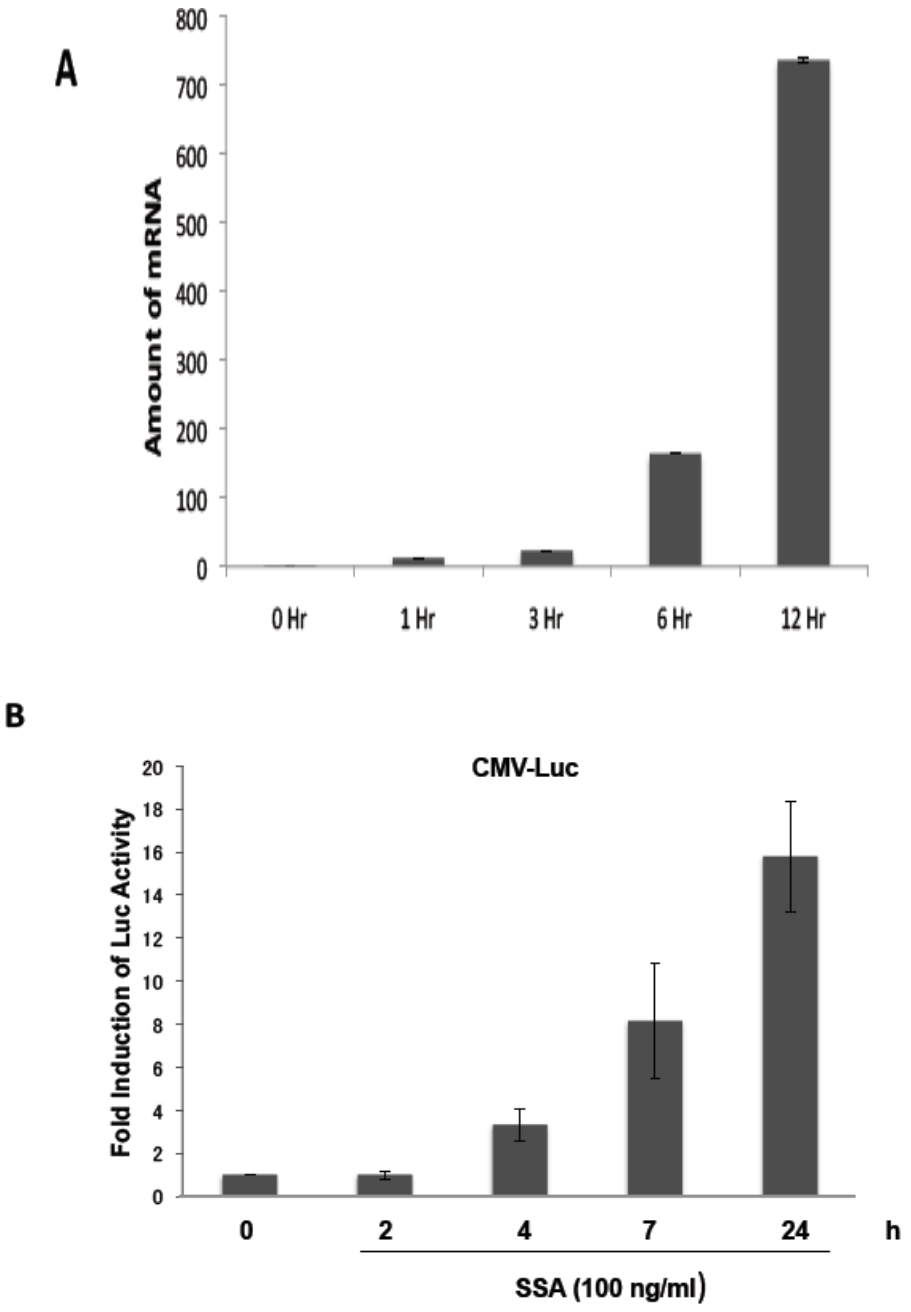


Figure 2.2. Time dependent CMV promoter activation by SSA.

(A) Time dependent CMV promoter activation was measured by qPCR and (B) Luciferase assay time course in the presence of SSA (100 ng/ml) in NCL-5 cells.

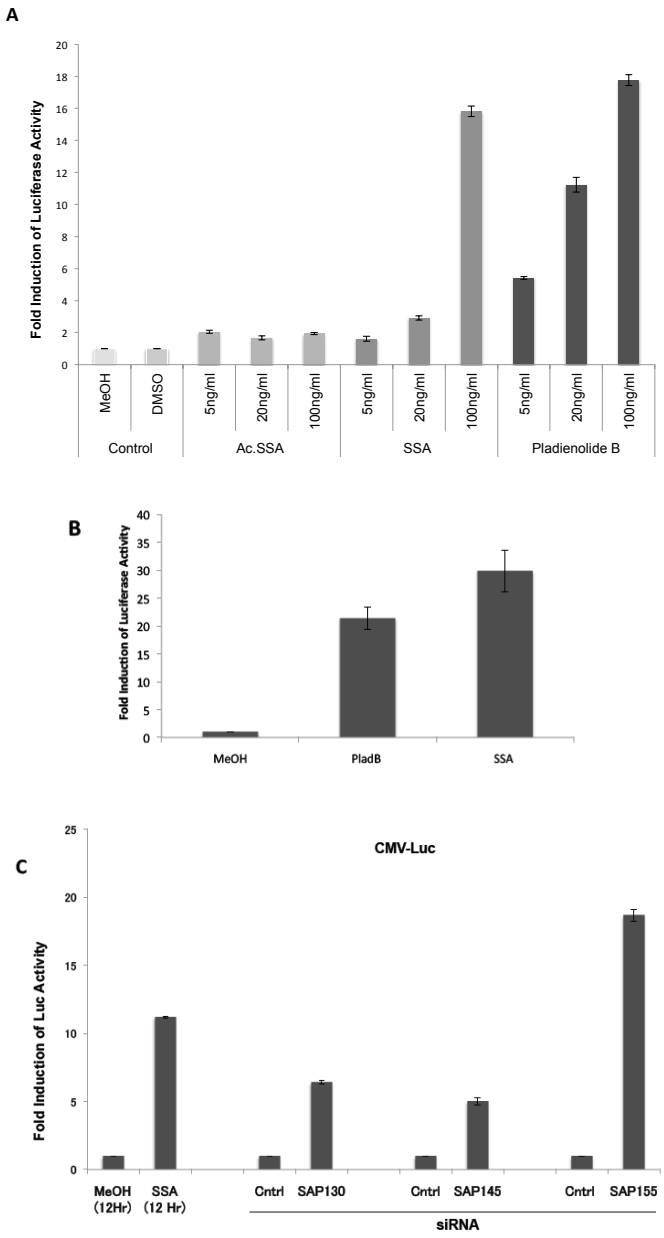


Figure 2.3.3. Effect of splicing inhibition on promoter activation.

(A) CMV promoter induction was quantified in NCL-5 cells in the presence of SSA, Plad B and Ac-SSA for 12 h luciferase assay. (B) NIH3T3 Mouse fibroblast cells were transiently transfected with the IL-8-Luc construct for 48 h followed by methanol, Plad B (100 ng/ml) and SSA (100 ng/ml) treatment for 12 h. IL-8 promoter activity was measured by luciferase assay. (C) Effect of SAP knockdown on CMV promoter induction. Components of SF3b, abbreviated, as SAP130, SAP145 and SAP155 were knocked down with small interference RNA for 96 h.

Chapter 3:
Role of ERK pathway in IL-8 and CMV promoter up-regulation

3.1: introduction

3.1.1: ERK pathway activation

Growth factors regulate gene expression of many proteins involved in preventing apoptosis by transmitting the signal from their receptors through Ras/Raf/MEK/ERK signaling pathway (18). Ras is the most upstream molecule of this pathway. Four Ras protein namely Ha-Ras, N-Ras, K-Ras 4A and K-Ras 4B have been identified. All Ras proteins activate Raf/MEK/ERK signaling pathway with varying potency. Binding of cytokines, growth factors or mitogens to their receptors usually activates the kinase activity of the cytoplasmic domain of the receptor. The activated cytoplasmic domain of receptor interacts with a complex Shc/Grb2/SOS, which leads to the activation of this complex. Activated SOS then removes the GDP from Ras family members, allowing Ras to bind with GTP for its activation. GTP-bound Ras can then recruit its downstream kinase Raf. Activated Raf phosphorylates and activates MEK1/2 and MEK1/2 then phosphorylates and activates the last kinase of the pathway, i.e. ERK1/2 (19).

3.1.2: Abnormal activation of ERK leads to disease development

Abnormal activation of this pathway occurs in acute myelogenous leukemia, acute lymphocytic leukemia, and breast and prostate cancer. Abnormally it is

activated by mutation in any protein of this pathway that eventually leads to constitutive activation of the protein and no longer requires

ligand for its activation. Its involvement in cancer gain considerable interest and scientist found the inhibitors of Ras, Raf, MEK and its downstream targets have been developed and many are in clinical trials.

3.1.3: Role in controlling apoptosis

Raf/MEK/ERK cascade is involved in prevention of apoptosis by regulating downstream transcription factors. Raf/MEK/ERK cascade phosphorylate BAD (Bcl-2-associated death) protein, which leads to its inactivation. Phosphoryaltion of BAD prevents its interaction with anti-apoptotic members of Bcl-2 family (Bcl-2 and Bcl-xL). On contrary, dephosphorylation favours the association of BAD with Bcl-2 and Bcl-xL and this interaction is pro-apoptotic. Ras/Raf/MEK/ERK cascade can phosphorylate anti-apoptotic Mcl-1 protein and the pro-apoptotic Bim protein. Phosphorylation of Bim results in its dissociation from Bcl-2, Bcl-XL and Mcl-1. Bim is degraded by proteasome that allow Bcl-2, Bcl-XL and Mcl-1 to bind to pro-apoptotic protein Bax, which make it inactive. Thus apoptosis is inhibited (20, 21).

3.1.4: Role in transcriptional activation

Extracellular-signal-regulated kinases 1,2 (ERK) are S/T kinases and can enter nucleus to phosphorylate many proteins involved in cell cycle regulation. Furthermore, it can directly phosphorylate many transcription factors including Ets-1, c-Jun, CREB and c-Myc. Moreover, ERK can phosphorylate inhibitor κB kinase (IKK) that lead to the activation of the NF-kB transcription factor. Besides this, in total ERK have more then 160 targets (22). In cells phosphatases are actively involved to remove the phosphate from phosphorylated ERK. So, ERK can be activated alternatively by inhibition of the phosphatase activity. ERK/MAPK pathway can phosphorylate histone H3

at ser 10 and ser 28, further effects the physical remodeling of chromatin structure that makes relax chromatin structure and open the DNA to be easily accessible by transcription factors (23).

3.1.5 SSA and ERK pathway

IL-8 is one of the well-studied CXC family chemokine's that activate, and accumulates leukocytes at the site of inflammation. Interleukin 1 (IL-1), Tumor necrosis factor alpha (TNF-α) and phorbol 12-myristate 13-acetate (PMA) are well known inducers of IL-8. These inducers can enhance the transcriptional stimulatory activity of viral promoter (SV40 and CMV) and are also able to activate signaling kinases. Previous investigations suggested that IL-8 production is regulated by extracellular-regulated protein kinase (ERK) in response to TNFα, IL-1β or PMA. To better understand the underlying mechanism I investigated possible upstream signaling pathways for IL-8 induction. The mitogen-activated protein kinases (MAPKs) are important signaling kinases that activate variety of transcription factors mediated through phosphorylation. Here I report that SSA-induced IL-8 and CMV promoters were associated with increased extracellular signal-related kinase (ERK) activation and were sensitive to PD98059.

3.2 Materials and Methods:

3.2.1 Materials, cell lines and antibodies

A549, NIH3T3, NCL-5 and Hela cells were maintained in DMEM with 10% FBS. NCL-5 cell line is a stable tranfected cell line with luciferase driven CMV promoter (Young Bae Kim et al, Oncogene. 1999). PD98059 was purchased from Wako Chemicals and Cell signaling technologies.

3.2.2 Antibodies and Immunoblotting

Antibodies against anti-phospho-ERK1/2 and anti-αERK were purchased from Cell Signaling Technology. Antibodies against luciferase (sc-32896), SAP145 (sc-101133), were purchased from Santacruz Biotechnology. Antibodies against SAP 130, SAP155 and β-actin were purchased from (Abcam). Mouse monoclonal anti α-Tubulin (B-5-1-2) antibody was from Sigma. Cells were lysed in NETN lyses buffer (50mM tris-Hcl pH7.5, 150mM NaCl, 5mM EDTA, 0.1% Nonidet P-40), containing 1% protease inhibitor Coktail (Roche) and protein concentrations were determined. Cell lysate was dissolved in 1XSDS–PAGE sample buffer and heated at 95 °C for 5 min. 20 μg of total proteins were resolved in 10% SDS–PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore) by electroblottting. Membranes were blocked in skim milk followed by incubation with primary and secondary antibodies and immune complexes detected by Immobilon™ Western Chemiluminescent HRP substrate (Millipore) in a LAS-3000 image analyzer (GE Healthcare).

3.2.3 qPCR analysis

Total RNA was prepared from drug treated cells with RNeasy Mini Kit (QIAGEN) and cDNA was prepared by reverse transcription with random 9-mer primers using RNA PCR Kit (AMV) Ver.3.0 (Takara Bio Inc., Shiga, Japan). Primers for target genes were designed by Universal Probe Library Assay Design Centre (Roche Applied Science) and gene expression was analyzed by qPCR SYBR Premix Ex Taq II (Takara BioInc.) and LightCycler 480 (Roche).

3.2.4 Expression and knockdown

NIH3T3 and A549 cells were seeded at $5x10^4$ cells per well in a 24 well multi-well plate and transfected with expression vectors using the Lipofectamine™ LTX reagent (Invitrogen) for 48 h and FuGENE® HD reagent (Roche) for 72 h. The pCR3.1-Luc represent wild type CMV promoter was kindly provided by Dr. Georg F. Weber (He B et al, BBRC. 2004). Deletion mutants and transcription factor point mutants of IL-8 were kindly provided by Dr. Naofumi Mukaida (Naoki Mori et al, Cancer Research. 1988). Cells were lysed in NETN buffer after treatment with drugs for indicated time points and luciferase activity was measured with SpectraMax L (Molecular Devices). Smal interference RNA (siRNA) for SAP-130, SAP-145, SAP-155 and control non-specific siRNA sequences were from Dharmacon.

3.3 Results:

3.3.1 SSA affects ERK activation.

As there is close relation between IL-8 gene expression and the ERK pathway and it has been reported that IL-8 inducers can enhance the transcriptional up-regualtion of viral promoters. To elucidate the role of ERK signaling pathway in IL-8 and CMV promoter stimulation, I examined the involvement of ERK in the SSA-induced promoter activation by immunoblotting with specific antibodies recognizing the phosphorylated form of ERK1/2. I found that SSA induced persistent time-dependent phosphorylation of ERK. Phosphorylation of ERK1/2 proceeds after 3 h and reaches maximum after 12 h as shown in (Fig. 3.1 A). As I sowed previously that SSA-induced IL-8 and CMV promoter activation initiates after 2-3 h, these results suggest a direct relation between transcriptional enhancement and ERK phosphorylation.

As it has been confirmed that SSA affects ERK1/2 phosphorylation, I developed a strategy to inhibit ERK1/2 phopshoryaltion by an ERK pathway inhibitor, PD98059. The NCL-5 cells were pretreated with 100 μM PD98059 for 1 h followed by incubation with SSA (100 ng/ml) for 10 h. The inhibition of pERK1/2 was confirmed by western blotting as shown in (Fig. 3.1 B-C). We followed the same strategy to elucidate the role of ERK1/2 in IL-8 promoter activity. A549 cell were transiently transfected with the luciferase reporter construct driven by IL-8 promoter for 48 h. A549 cells were pre-incubated with PD98059 for 1 h followed by SSA treatment. Cell lysate was collected in NETN lysis buffer and protein concentration was determined by Bradford assay. The IL-8 promoter activity was confirmed by luciferase assay as shown in (Fig. 3.1 D). Both promoters were found to be sensitive to pERK1/2 inhibition.

Furthermore, we found that promoter induction of CMV promoter was inhibited upon addition of PD98059 in a dose-dependent manner (data not shown). Addition of PD98059 inhibited pladienolide B-induced activation of

IL-8 promoter as well as shown in (Fig. 3.1 E). The inhibition of IL-8 and CMV promoter activity was confirmed by the inhibition of luciferase via luciferase assay. These results suggest an involvement of the ERK pathway in CMV and IL-8 promoter induction.

3.3.2 Effect of SAP knockdown on CMV promoter activity

SSA specifically binds to a subunit of U2 snRNP called SF3b to inhibit splicing. A typical SF3b is composed of SAP130, SAP145, SAP149, SAP155 and p14. We showed previously that SAP proteins knockdown enhanced CMV promoter transcriptional activation. As we know that SSA-induced activation of CMV and IL-8 promoters is ERK pathway dependent, we aim to see a link between SAP knockdown and ERK activation. I introduce small interference RNA targeting SAP130, SAP145 and SAP 155 into NCL-5 cells according to manufacturer instructions. After 96 h incubation, cells were collected in NETN lysis buffer, sonicated and the cell lysates were prepared. The protein concentration was determined by Bradford assay. Equal amount of proteins was resolved on 10% PAGE. Immunoblot analysis confirmed that the SAP knockdown induces the ERK pathway activation as shown in (Fig. 3.2 A)

3.4 Conclusion

Current data suggest that splicing inhibition and ERK activation are interrelated in terms of gene activation and ERK pathway activation. As ERK can activate many target transcription factors, it is involved in transcriptional activation of many genes. Our data clearly indicate that splicing inhibition induces ERK activation, thereby up-regulating the IL-8 and CMV promoters, although we do not know their target transcription factors of ERK at this stage.

Figures Chapter 3:

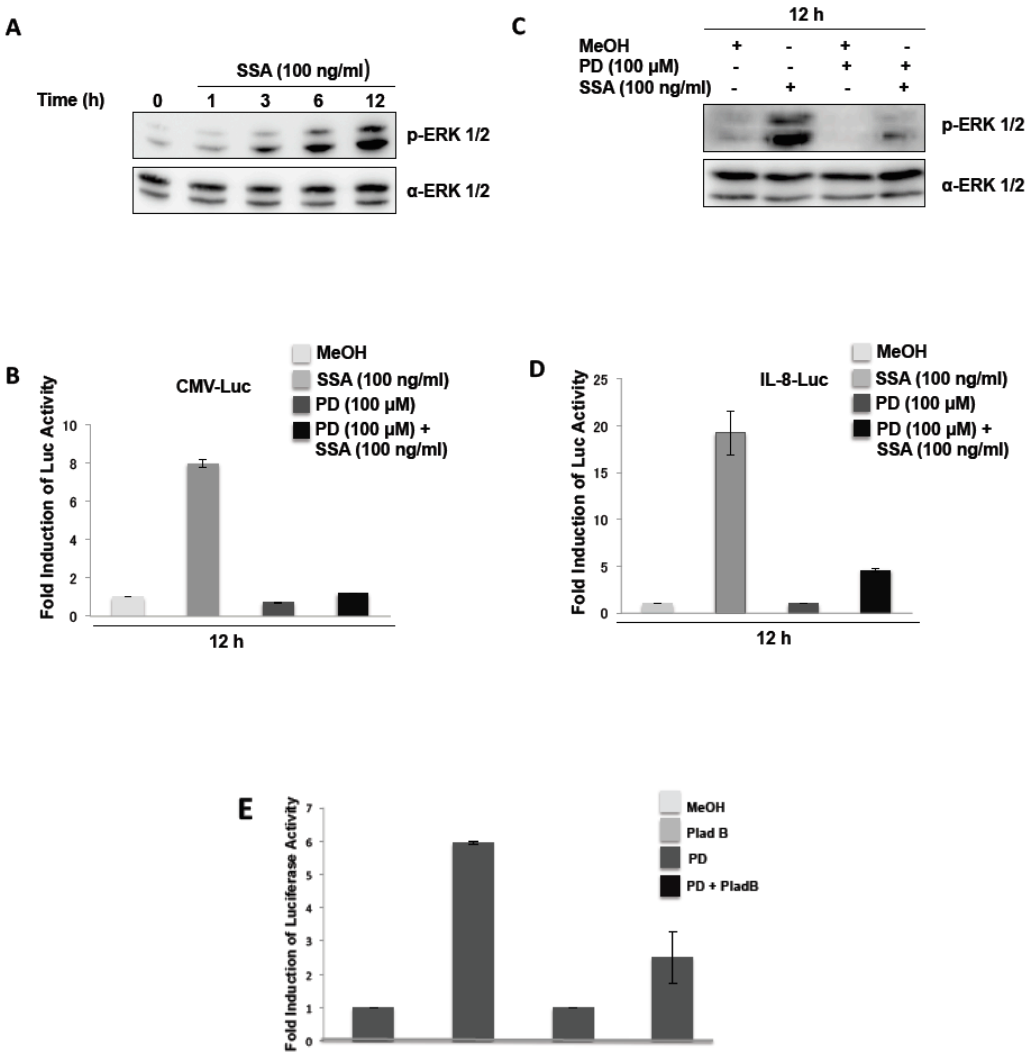


Figure 3.1. Activation of the ERK signaling pathway.

(A) SSA treatment led to increased ERK activation over time in NCL-5 cells, an NIH3T3 cell line stably transfected with a CMV reporter. (B) Treatment with PD also inhibited expression of the CMV promoter in NCL-5 cells (C) The MEK inhibitor PD098059 (PD) reduced SSA-dependent ERK-phosphorylation. (D) PD inhibited IL-8 promoter in transiently transfected A549 cells upon SSA treatment and (E) plad B as well. Error bars represent standard error of mean.

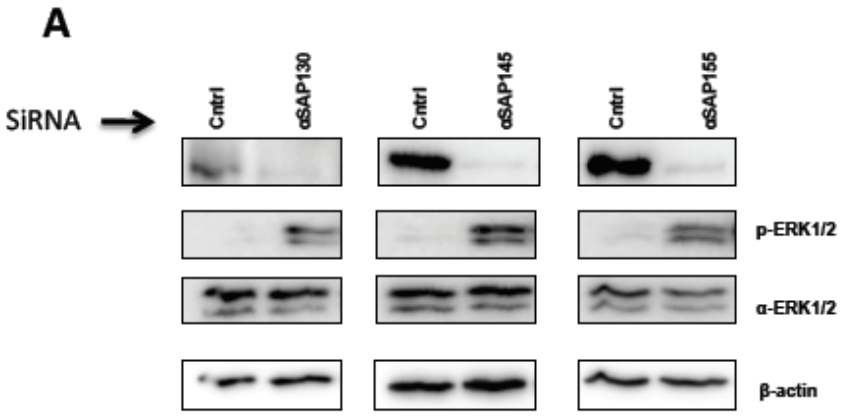


Figure 3.2. (A) Knockdown of key components of the SF3b subcomplex, activates ERK signaling pathway in NCL-5 cells.

Chapter 4

SSA affects NF-kB pathway

4.1: Introduction

The nuclear factor-kB (NF-kB) family of proteins is extensity-studied protein (24). Among a variety of transcription factors, NF-kB plays a key role in the expression of genes involved in immune and inflammatory responses, cell growth, proliferation and survival (25, 26). The dysregulation of NF-kB pathway leads to inflammatory disorders, cancer and autoimmune and metabolic diseases (27, 28, 29, 30). The NF-kB always exist in homodimer or heterodimer form from five subunits; p50, p52, RelA (p65), cRel and RelB (24). In response to inducers that includes physical, physiological and oxidative stress, the two main NF-kB heterodimers p50: RelA and p52: RelB are rapidly activated (31). These dimers once activated binds to the κB sites in promoters of variety of genes (32, 33). Under inactive condition, NF-kB dimers are bound to inhibitory proteins called IκB (IκBα or IκBβ) proteins. The inducers of NF-kB pathway initiate the phosphorylation of IκBs by IκB kinases called IKKα and IKKβ. The phosphorylated IκB proteins are subjected to ubiquitination and then proteosomal degradation that ultimately release NF-kB dimers to translocate to the nucleus (34). In cells, NF-kB is activated through canonical and non-canonical pathways. The canonicals pathway depends on IKKβ and NEMO mediated phosphorylation of IκBα and nuclear translocation of p65. In contrast, the non-canonical pathway depends on IKKα to phosphorylate p100 and leads mainly to the nuclear translocation of p52 (34).

To better understand the underlying mechanism I investigated possible transcription factors responsible for IL-8 induction. The well-known activators of IL-8 such as TNFα, IL-1β or PMA specifically target ERK and also NF-kB transcription factor to modulate IL-8 gene activation. Next I design an approach to look for target transcription factor with in IL-8 gene.

The promoter region of IL-8 contains a putative nuclear factor-kappa B (NF-kB), activator protein-1 (AP-1) and CCAAT/enhancer-binding protein (C/EBP) binding sites (35). Role of transcription factor in IL-8 activation was confirmed by site-directed mutagenesis of each transcription factor-binding site in IL-8 promoter region. Mutation of NF-IL6 and AP-1 site had no effect upon SSA treatment. Mutation of NF-kB site reduced SSA-stimulated expression of IL-8 gene. The results from mutation of NF-kB binding sites were consistent with small interfering RNA (siRNA) silencing of p65 subunit of NF-kB and parthenolide (NF-kB pathway inhibitor). Our data confirm the central and essential role of NF-kB in up-regulation of IL-8 gene upon SSA treatment.

My Current work suggests the activation of IL-8 and CMV promoter through canonical NF-kB pathway by a bacterial product, SSA. Another splicing inhibitor, pladienolide B work in a similar way thus further supported this idea.

4.2. Material and Methods

4.2.1 Materials, cell lines and antibodies

A549 and NIH3T3 cells were maintained in DMEM with 10% FBS.
Parthenolide was purchased from Wako Chemicals.

4.2.2 Antibodies and Immunoblotting

Antibodies against NF-kB-p65 (sc-109) and IκBα (sc-371) were purchased from Santacruz Biotechnology. Cells were lysed in NETN lyses buffer (50mM tris-Hcl pH7.5, 150mM NaCl, 5mM EDTA, 0.1% Nonidet P-40), containing 1% protease inhibitor Coktail (Roche) and protein concentrations were determined. Cell lysate was dissolved in 1XSDS–PAGE sample buffer and heated at 95 °C for 5 min. 20 μg of total proteins were resolved in 10% SDS–PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane (Millipore) by electroblottting. Membranes were blocked in skim milk followed by incubation with primary and secondary antibodies and immune complexes detected by Immobilon™ Western Chemiluminescent HRP substrate (Millipore) in a LAS-3000 image analyzer (GE Healthcare).

4.2.3. Immunofluorescence Microscopy

Cells grown on coverslip, were treated with vehicle control or SSA (100 ng/mL) for indicated time points followed by 15 min fixation in 4% paraformaldehyde. Cells were then permeabilized in 0.2% Triton X-100 in PBS (PBT), followed by blocking in 0.1% PBT containing 5% normal goat serum (PBTN) for 15 min. Immunostatining was performed with anti- NF-kB – p65 primary antibody (1:200 dilution), for 1–2 h followed by the Alexa Fluor 594, secondary antibody (Invitrogen) for 20 min. Slides were mounted with mounting medium (Vector Laboratories), and images were taken using a Delta Vision Microscope (SEKI Technotron Corp.).

4.2.3 Expression and knockdown

NIH3T3 and A549 cells were seeded at $5x10^4$ cells per well in a 24 well multi-well plate and transfected with expression vectors using the Lipofectamine™ LTX reagent (Invitrogen) for 48 h and FuGENE® HD reagent (Roche) for 72 h. The pCR3.1-Luc represent wild type CMV promoter was kindly provided by Dr. Georg F. Weber (He B et al, BBRC. 2004). Deletion mutants and transcription factor point mutants of IL-8 were kindly provided by Dr. Naofumi Mukaida (Naoki Mori et al, Cancer Research. 1988). Cells were lysed in NETN buffer after treatment with drugs for indicated time points and luciferase activity was measured with SpectraMax L (Molecular Devices). Small interference RNA (siRNA) for NF-kB-p65 was from Nippon Gene.

4.3 Results

4.3.1 Transcription factor point mutant's reveals an essential role of NF-kB in the regulation of IL-8 and CMV promoters.

The cis-element for IL-8 gene activation was identified by various 5′ deletions of IL-8 promoter as shown in (Fig. 4.1 A). NIH3T3 cells were transiently transfected for 48 h, followed by incubation with 100 ng/ml SSA or without SSA. Activity of each construct was quantified and measured by luciferase assay. SSA enhances the luciferase activity of cells transfected with -1481, -272 and -133 5′ deletions constructs to similar extent. A slight decrease in the luciferase activity was found in -98 but -50 5′ deletion construct abolished SSA stimulatory affects drastically as shown in (Fig. 4.1 B). For further experiments I used -133bp construct as wild type. These results clearly suggesting that SSA responsive elements are residing within 3′ regions downstream of -133 bp construct.

Positions of transcription factor binding sites in -133bp construct of interleukin-8 promoter are described in (Fig. 4.1 C). To investigate the regulatory role of each site, I used transcription factor site mutant construct for each transcription site as shown in (Fig. 4.1 D). These constructs were introduced into NIH3T3 transiently for 48 h followed by addition of SSA and without SSA. The level of each construct lacking transcription factor binding sites were measure through luciferase assay and luminescence was compared with wild type. Current data suggest that NF-kB site mutant severely reduced the induction of luciferase activity upon SSA treatment. I further demonstrate that NF-kB transcription factor point mutant of IL-8 was found resistant to pladienolide B treatment as well as shown in (Fig. 4.1 E). While the mutation of NF-IL6 synergistically enhance the activity of IL-8 promoter activity in response to SSA. NF-IL6 belongs to family of C/EBP, which contain basic-leucine zipper (bZIP) domain at the C-terminus that facilitates the formation of homodimers and heterodimers with other leucin

zipper containing proteins (36, 37). Previous reports showed that NF-IL6 and NF-kB are in close proximity with each other in enhancer region of IL-8 (38). Stein et al, demonstrated in experiment that used IL-8 promoter construct with cotransfection of NF-IL6 expression vector. The NF-IL6 expression vector was found to inhibit IL-8 stimulation (39, 40). So, the authors assume that mNF-IL6 construct might have more space for binding of NF-kB transcription factor to its target site. Our results are in consistence with Stein et al hypothesis and other past findings using same constructs (41). Role of NF-IL6 in IL-8 promoter stimulation seems to be controversial with other previous findings. They could not observe any affect of NF-IL6 site mutant on IL-8 promoter induction (42, 43, 44).

4.3.2 SSA modulates the transcriptional activity through p65-NF-kB

The NF-kB activation takes place following the phosphorylation of IκBα and IκBα phosphorylation results in ubiquitination and rapid degradation of IκBs by proteasome, allowing NF-kB to translocate to the nucleus (45). It has been reported that NF-kB is tightly bound to IκBα that retained NF-kB in cytoplasm (46). These result shows that SSA might affect the nuclear translocation of p65 component of NF-kB to activate IL-8 gene. The nuclear translocation was confirmed by immunostaining p65 component of NF-kB as shown in (Fig. 4.2 A). The nuclear translocation of p65 indicated that it has been dissociated from the IκBα complex. I check the degradation of IκBα upon SSA treatment. I observed time dependent degradation of IκBα upon SSA treatment. The degradation of IκBα initiates after 2 to 3 h. it's consistent with EKR1/2 phosphoryaltion and promoter activation results as degradation of IκBα initiates at same time interval.

To further elucidate the role of IκBα degradation and NF-kB activation, it is necessary to analyze IL-8 induction by preventing IκBα degradation. The commonly used drug to prevent IκBα degradation is MG132. The mode of action of MG132 is to inhibit proteasome, which is actively involved in IκBα degradation. The IL-8 and CMV promoters have AP-1 binding sites. The

mitogen-activated c-Jun N-terminal kinase (JNK) activates c-Jun, which is a component of AP-1 transcription factor. It has been reported that MG132 transactivate IL-8 and CMV promoters via AP-1 transcription factor. I found synergistic activation of CMV promoter upon MG132 and SSA addition (data not shown). I found the time dependent degradation of IκBα as shown in (Fig. 4.2 B), which means that SSA affects NF-kB pathway and dissociated p65 from IκBα and hence it has been translocated to nucleus.

Alternatively I prefer to block the upstream activator of NF-kB pathway. The NF-kB pathway inhibitor parthenolide that specifically inhibits the phosphorylation of IκBα by upstream kinases, eventually prevent its degradation via proteasome. The promoter activity of IL-8 and CMV was assessed by 1 h pre-treatment of A549 cells transiently expressing luciferase gene under the control of IL-8 promoter and NCL-5 cells stably expressing luciferase gene under the control of CMV promoter with 40 μM parthenolide. I found that 40 μM parthenolide inhibit SSA-induced IL-8 and CMV promoter activity as shown in (Fig. 4.2 C-D). Parthenolide rescue the degradation of IκBα and was confirmed by western blot by using anti- IκBα antibody as shown in (Fig. 4.2 E)

Current data suggest that only NF-kB transcription factor-binding site was largely inhibitory in response to SSA, furthermore it was confirmed by rescuing IκBα degradation with parthenolide. The role of AP-1 site is not very significant. Parthenolide has been reported to affect AP-1 transcription factor binding to its target site by dephosphorylating JNK. There might be other unknown side effect of parthenolide. Alternatively, I prefer to observe the effect p65 knockdown on SSA-induced IL-8. A549 cells were transiently co-transfected followed by small interference RNA for 72 h. The IL-8 promoter activity was confirmed by luciferase assay in the presence or absence of SSA as shown (Fig. 4.2 F). The siRNA against p65 inhibits IL-8 promoter activation upon SSA treatment. The knockdown efficiency was confirmed by western blot by using anti-p65 antibody as shown in (Fig. 4.2 G).

Conclusion:

I demonstrated that SSA-induced IkBα/nuclear translocation of p65-NF-kB is required for CMV and IL-8 promoters activation. I demonstrated that SSA-induced NF-kB pathway enhance the transcriptional up-regulation of anti-apoptotic NF-kB target gene, A-20 (data not shown). Furthermore, NF-kB regulates variety of genes involve in regulation of cytokines, growth factors, adhesion molecules, intracellular signaling molecules, transcription factors and miRNAs. In cancer, activated NF-kB pathway is associated with tumor promotion and progression and also makes resistance cancerous cells to chemotherapy and radiotherapy. Prevention of NF-kB pathway activation is very important for cancer therapy.

Figures Chapter 4:

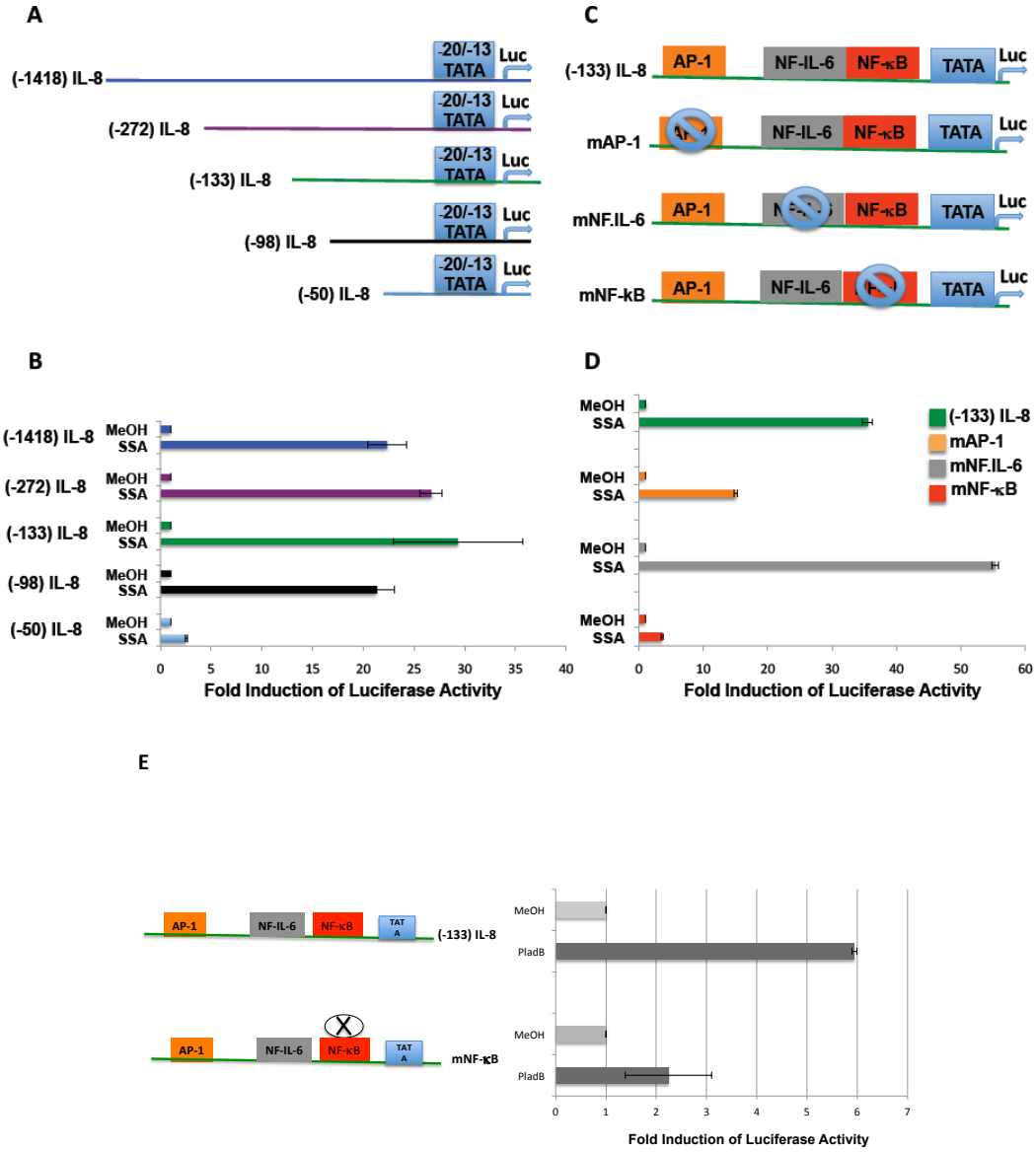


Figure 4.1. Identification of the transcription factor responsive to SSA treatment. (A) 5′ Deletion mutants of the IL-8 promoter that were introduced into NIH3T3 cells. (B) Luciferase activity of 5′ deletion mutants of IL-8 was measured after 48 h post transfection upon 12 h SSA treatment. (C) Diagram outlining deletion mutants of the IL-8 core promoter, lacking individual transcription factor binding sites. (D) Comparison of SSA-responsiveness of above mutants. Constructs without the NF-kB binding site become unresponsive to SSA-induced promoter activation. (E) Construct without NF-kB binding site become unresponsive to plad B-induced IL-8 promoter activation.

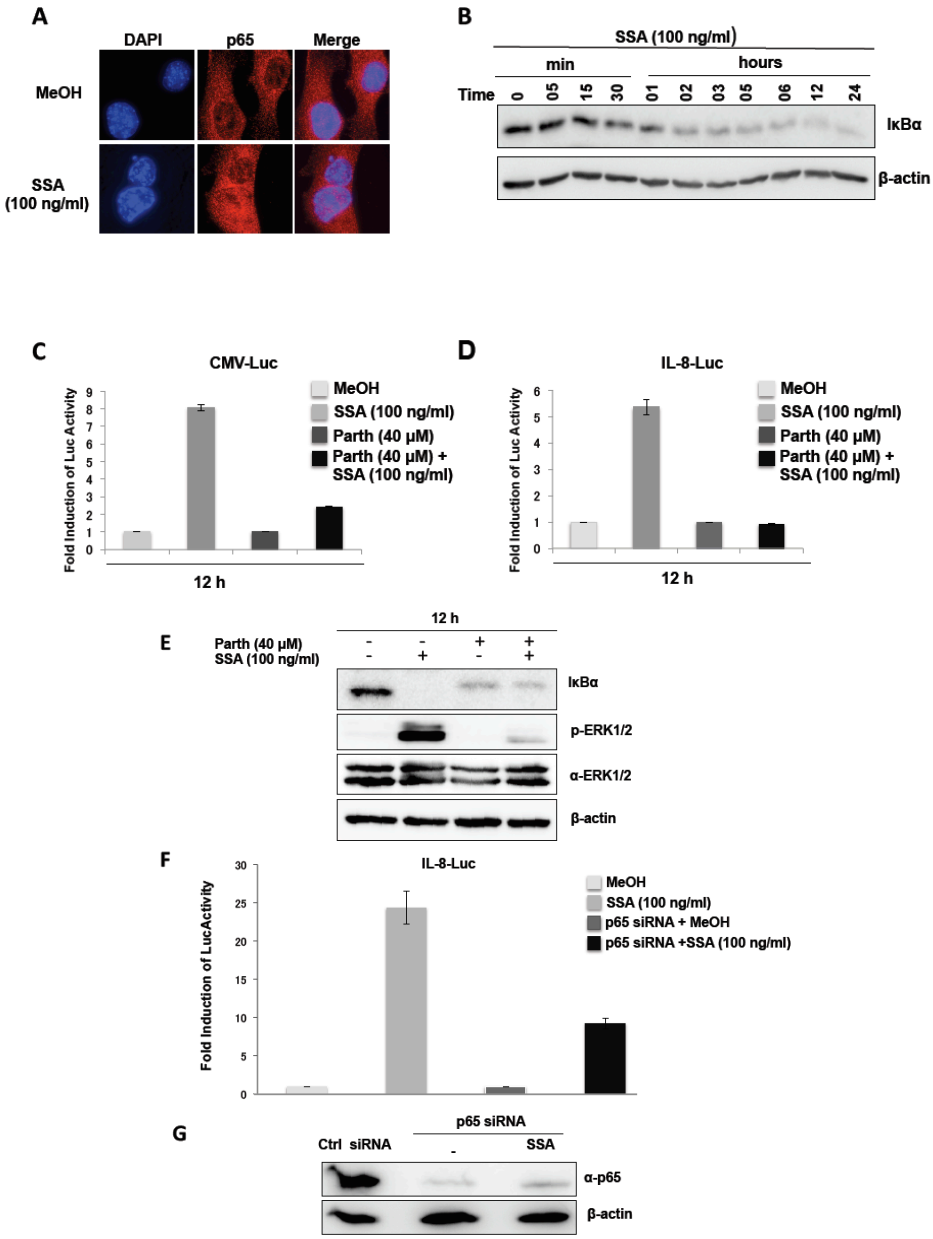


Figure. 4.2. SSA treatment leads to NF-kB activation. (A) NIH3T3 cells were treated with either control (upper panel) or SSA (100 ng/ml) (lower panel), nuclear localization of the p65 subunit was determined by immunostaining. (B) Cellular amounts of IκBα were determined by western blot at different time intervals. (C) Levels of IL-8 and (D) CMV promoter activation by SSA (100 ng/ml) were determined by luciferase reporter assay in transiently transfected A549 cells in the presence and absence of 40 μM parthenolide. (E) 40 μM parthenoliod tratment rescue IκBα degradation and ERK phosphorylation. (F) p65 knockdown inhibits the SSA-induced IL-8 induction.

Chapter 5

SSA induces ER stress

5.1 Introduction

5.1.1 what is ER stress?

In the endoplasmic reticulum secretory and transmembrane proteins are synthesized, processed and folded with the help of ER chaperons. Polypeptides after entering the ER through the SEC61 translocator are folded and postranslationaly modified (for example disulphide bond formation and N-linked glycosylation) (46). The lumen of the ER is rich in Ca^{2+}, Ca^{2+}-dependent chaperons and calreticulin, which together generate an oxidative environment that is favorable for disulfide bond formation and proper protein folding (48, 49). For normal cellular activities and survival, the efficient functioning of the endoplasmic reticulum is necessary. Environmental and genetic factors that interfere with normal ER function lead to the accumulation and aggregation of missfolded or unfolded proteins. This process triggers the so-called Unfolded Protein Response (UPR) (50). The role of UPR is to reestablishnormal ER function by enhancing the folding capacity of ER and promoting ER-associated protein degradation (ERAD) to dispose of malfolded proteins. When the amount of aggregates exceeds the folding capacity of ER, the condition is termed as ER stress (51). Protein aggregation is toxic to cells; consequently, ER stress activates a complex signaling network to reduce the burden on the ER. If ER stress persists for long time, apoptotic cell death occurs (16, 35).

5.1.2 Cellular responses combating ER stress

There are three cellular responses to combat ER stress mediated via ER transmembrane receptors: pancreatic ER kinase (PERK), activating transcription factor 6 (ATF6) and inositol-requiring enzyme 1 (IRE1). Under

normal conditions they are associated with ER chaperon GRP78 (Bip). But when missfolded proteins accumulate, GRP78 releases these transmemberane-signaling molecules launching the UPR (52).

PERK is activated through autophosphorylation after being released from GRP78. The activated PERK phosphorylates, eukaryotic translation initiation factor 2 (eIF2). Inhibition of translation decreases the burden on ER and ultimately increases cell survival. ATF6 is expressed in two forms, ATF6α and ATF6β. ATF6α has a cytoplasmic domain that contains a bZIP motif and acts as a transmembrane transcription factor as well as an ER-luminal domain, which contains a Golgi-localization sequence. On onset of ER stress, ATF6α dissociates from BIP and is translocated into the Golgi via its ER-luminal domain. Inside the Golgi apparatus, it is cleaved by site-1 proteases (S1P and S2P), and its cytoplasmic domain is separated, which then translocates to the nucleus where it participates in activating the transcription of target genes (53, 54). ATF6β has a DNA binding domain but plays a minimal role in UPR target gene activation.

IRE1 is expressed in two isoform, IRE1α and IRE1β (55). Dissociation from GRP78 activates its both domains, which are, a cytoplasmic domain that contains serin/threonine kinase and a C-terminal endoribonuclease domain (55, 56, 57). Once activated, the cytoplasmic domain of IRE1 interacts with tumor-necrosis factor (TNF)-receptor-associated factor 2 (TRAF2), that activates apotosis signal regulating kinase 1 (ASK1) (58) which ultimately activates downstream JUN N-terminal Kianse (JNK) (59). This allows the cells to eliminate damaged organelles via autophagy (60). JNK might promote apoptosis through caspase-12 (61). The kinase activity is less well studied compared to the endoribonuclease activity of IRE1. The activated IRE1 post transcriptionally processes the X-box-binding protein 1 (XBP1) mRNA by splicing that ultimately excises a 26-nucleotide sequence from XBP1 mRNA. This splicing is different from traditional mRNA splicing mechanisms, and causes a frame shift mutation, which generates spliced variants called XBP1s and XBP1u. The XBP1s protein is 376 amino acids long and activates its target gene in the nucleus upon translocation. The target genes activated by

XBP1-s assist in protein synthesis and secretion. Besides this, XBP1-s increases lipid biosynthesis and ER biogenesis. On other hand, XBP1-u has 261 amino acids and is a short-lived protein that is degraded both in an ubiquitin-dependent and ubiquitin-independent manner (62). Like XBP1-s, XBP1-u has a nuclear localization signal but lacks a transactivation domain and is thus unable to activate UPR-dependent genes (63). XBP1-u under certain conditions suppresses the activity of XBP-1s by inhibiting its nuclear translocation (64).

The formation and rearrangement of disulfide bonds often rely on catalysis of thiol-disulfide oxidoreductases. Thiorodoxine related memeberane protein (TMX) and protein disulphide isomerase (PDI) are localized in the ER and are involved in cellular redox regulation. TMX can bind to calnexin (membrane bound chaperon), which might be important for its retention on the ER membrane 65). PDI is well characterized and is believed to catalyze the disulphide bond formation of substrate proteins through thiol-disulfide exchange (66). An experiment with tagged TMX showed its ER localization and overexpression relieve ER stress induced by brefeldin (BFA), an inhibitor of ER-Golgi transport (65). The TMX possesses N terminal signal peptide, one Trx-like domain with unique active site sequence, CXXC and a transmemberane domain. The XX represent proline and alanine respectively.

5.1.3 FR901464 as an ER stress inducer

Etsu Tashiro identified TMX as FR-binding protein using biotinylated SSA. He isolated possible FR-binding proteins, in HeLa cell extracts and identified TMX as FR-binding protein (unpublished data). Both N-terminally GST-fused TMX expreseed in *E. coli* and C-terminally myc-epitoped TMX expressed in 293T cells could bind to biotinylated SSA *in vitro* and *in vivo*. Since the TMX/SPAC mutant in whom Cys-56 in the thioredoxin-like domain was exchanged for serine could not form a complex with FR. This suggested that FR bound to TMX via Cys-56 in the thioredoxin-like domain, likely via a covalent bond. Furthermore, FR induced ERSE-dependent Bip promoter activation and lead

to increase frame switch splicing of XBP-1 while also inducing JNK phosphorylation. Although TMX knockdown by siRNA resulted in activation of the Bip promoter and elevation of frame switch spliced XBP-1 protein, FR could not increase Bip promoter activation or frame switch spliced XBP-1 expression in TMX knockdown cells. Taken together of these results, FR appears to induce ER stress via TMX.

Tunicamycin and thapsigargin are well-known inducers of ER stress. It has been reported that ER stress inducers can activate the NF-kB pathway, which will eventually lead to up-regulation of NF-kB target genes. I further found that thapsigargin induced IL-8 promoter activity and up-regulated NF-kB target genes. To identify the involvement of ER stress in gene activation, I first confirmed the binding of biotinylated SSA to TMX by using biotinylated SSA and found that SSA also binds to TMX. Then I knocked down TMX with small interference RNA in A549 cells and treated these cells with SSA. SSA was found to enhance IL-8 promoter activity in TMX-knockdown cells as well clearly suggesting SSA stimulatory effect is independent of its binding to TMX and ER stress induction.

5.2 Material and Methods

5.2.1 Materials, cell lines and antibodies

HeLa and A549 cells were maintained in DMEM with 10 % FBS. Thapsigargin was purchased from Wako Chemicals. The following antibodies were purchased from Cell Signaling: anti-phospho-JNK, anti-JNK, and HRP-conjugated anti-biotin.

5.2.2 Invitro binding assay

HeLa cells were extracted in lysis buffer containing 50 mM HEPES (pH=7.6), 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 1 mM DTT, 1 mM NaF, 10 % Glycerol, 0.1 % Tween-20, 1 % NP-40, 0.1 mM PMSF and Protease Inhibitor Cocktail (Roche). For detection of biotinylated SSA-binding proteins, 4 mg of HeLa extracts were incubated with 1 nmol of biotinylated FR for 4 h at 4^{o}C, and then the biotinylated SSA-binding proteins were purified by incubation with streptavidin-agarose for 1 h at 4^{o}C. The streptavidin-agarose purified fraction was separated by SDS-PAGE using 5-20 % gradient gel and detected by HRP-conjugated anti-biotin antibody.

5.2.3 RNA interference

Small interfering RNA duplexes (siRNA) for TMX were synthesized by Dharmacon. The target sequence of TMX siRNA was 5'-AACUGCUGGAAGGAGACUGGA-3' (nucleotides 122-142 of human TMX). A549 cells were seeded on 24-well plates at a density of 3x10^{4} per well and the next day TMX siRNA were transfected using Dharmofect. After 24 h, cells were transfected again with the IL-8 promoter containing plasmid. After 48 h cells were treated with 100 ng/ml SSA for 12 h and then the luciferase activity of IL-8 promoter was measured by luciferase assay.

5.3 Results

5.3.1 FR induces UPR

Etsu Tashiro, a former member of our laboratory, discovered and additional protein that bound to FR901464 (in *vitro* in HeLa cell extracts. FR-binding proteins were identified using biotinylated SSA (Methylated derivative of FR) and analyzed by Western blotting using an anti-biotin antibody and four biotinylated SSA-binding proteins were detected whose molecular weights were 59, 48, 38.5 and 28 kDa, respectively. To investigate which protein was a relevant target of FR, he tested which protein could be competed away with free FR. He found that the 38.5 biotinylated SSA-biding protein competed with FR. His result suggested that the 38.5 kDa biotinylated SSA-binding protein is a primary target of FR/SSA. Therefore, he determined the protein's identity by LC-MS/MS analysis. The protein was identified as thioredoxin transmembrane protein (TMX).

Next he investigated whether FR induced UPR or not. The transcriptional activation of the Bip promoter is a well-characterized UPR marker. He cloned the human Bip promoter region from HeLa genomic DNA and subcloned it into the pGL3-Basic luciferase vector (designated Bip (-304/+7)). The Bip (-304/+7) plasmid was transiently introduced into 293T cell for 24 h, and after 24 h, the transfected cells were treated with 100 ng/ml of FR901464, 10 mg/ml of tunicamycin (Tm) and 1mM of thapsigargin (Tg) (agents that promotes UPR) for 12 h. FR901464 induced Bip (-304/+7) promoter activation at the same level as other ER stress inducing agents (Fig. 5.1 A). Previous reports showed that the Bip promoter contains three functional ERSEs (endoplasmic reticulum stress elements) and deletion of these three functional ERSEs abolished ER stress-induced Bip promoter activation. To examine whether FR901464-induced Bip promoter activation indeed resulted from UPR, the three ERSEs were mutated on the Bip promoter (designated Bip (-304/+7)/mERSE)) and its promoter activity was analyzed. As previously reported, Tm or Tg did not induce Bip (-304/+7)/mERSE promoter activation

in Bip (-304/+7)/mERSE transiently transfected 293T cells. FR901464 also failed to activate the Bip (-304/+7)/mERSE promoter.

To obtain more evidence that FR901464 induces UPR, XBP-1 frame switch splicing and JNK activation were examined. IRE1a has an endoribonuclease activity to excise an intron from the mRNA of the transcription factor XBP-1. This splicing event results in the conversion of a 267-amino acid XBP-1u encoded by unspliced XBP-1 mRNA into a 371-amino-acid XBP-1s. Thus, he examined whether FR901464 induced IRE1a-dependent XBP-1 frame switch splicing assessed by Western blotting using an anti-XBP-1 antibody. When HeLa cells were incubated with 100 ng/ml of FR901464, JNK was activated in a time- dependent manner. FR901464 also decreased unspliced XBP-1 (XBP-1u; 276a.a.) and increased frame switch spliced XBP-1 (XBP-1s; 371a.a.) (Fig. 5.1 B). When HeLa cells were incubated with 100 ng/ml of FR901464, JNK phosphorylation occurred after 9 h of FR901464 addition.

5.3.2 Identification of SSA binding proteins and activation of JNK

I tried to replicate Dr. Tashiro's experiment and could also identify a 38.5 kDa SSA-binding protein. It seems that SSA targeting TMX can activate UPR. Furthermore I detected the activation of JNK upon SSA treatment and compared the immunoblots with FR and pladienolide B. I found that pladienolide behaves similarly to FR while Ac-SSA fails to phosphoryalte JNK. Thus, I could confirm that TMX is one of the binding targets of SSA and pladienolide B and that SSA activates UPR. These results (Fig. 5.2 A-B) suggest the involvement of TMX in the induction of ER stress by SSA and pladienolide B.

5.3.3 SSA-induced IL-8 promoter activation occurs independent of TMX.

SSA is known to activate the IL-8 promoter and it is also well established that ER stress inducers can activate the NF-kB pathway. Thus, I examined whether ER stress inducing agent, thapsigargin (Tg) can also activate the IL-8 promoter and other NF-kB target genes. I found that Tg increased the mRNA of IL-8 and other NF-kB taget genes such as COX-2, SOD-2 and NOD-1 as shown in (Fig. 5.3 A).

Furthermore, I examined whether TMX knockdown by TMX siRNA activates the IL-8 promoter or not. An IL-8 promoter plasmid was co-transfected with TMX siRNA or non-specific siRNA into A549 cells. After 48 h transfection, the luciferase activity of IL-8 promoter was measured. The TMX knockdown by TMX siRNA did not inhibit IL-8 promoter induction. Therefore it appears that IL-8 promoter activation by SSA is independent of SSA binding to TMX as shown in (Fig. 5.3 B).

5.4 Conclusion

I confirmed that TMX is a target of SSA by using biotinylated SSA. To obtain more evidence that SSA induced UPR via binding to TMX thereby inhibiting TMX activity, I knocked down endogenous TMX by siRNA. When endogenous TMX was knocked down, I could not observe any increase in IL-8 promoter activity. Thus I concluded that TMX is one of the binding targets of SSA but it activates the IL-8 gene promoter independent of binding to TMX or simply SSA activates ERK and NF-kB pathway independent of binding to TMX.

Figures Chapter 5:

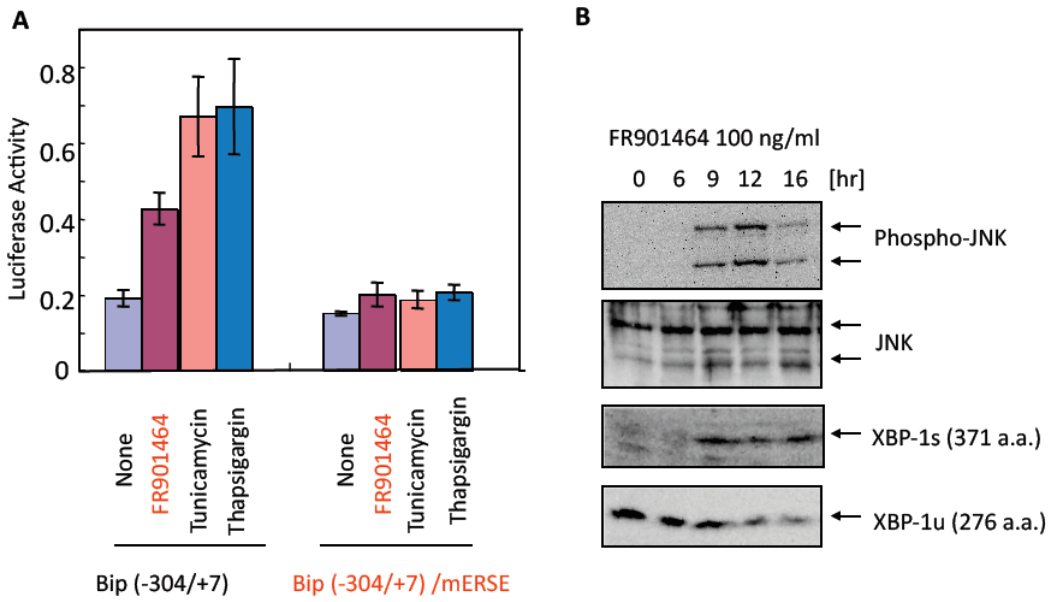


Figure 5.1: FR901464 induces ER stress. (A) 293 T cells were transiently transfected with Bip promoter containing constructs. Luciferase activity of wild type Bip and mutant Bip (with three deleted ERSEs) was measured in the presensce of FR and ER stress inducers. (B) Time dependent activation of JNK and converstion of XBP-1u into XBP-1s.

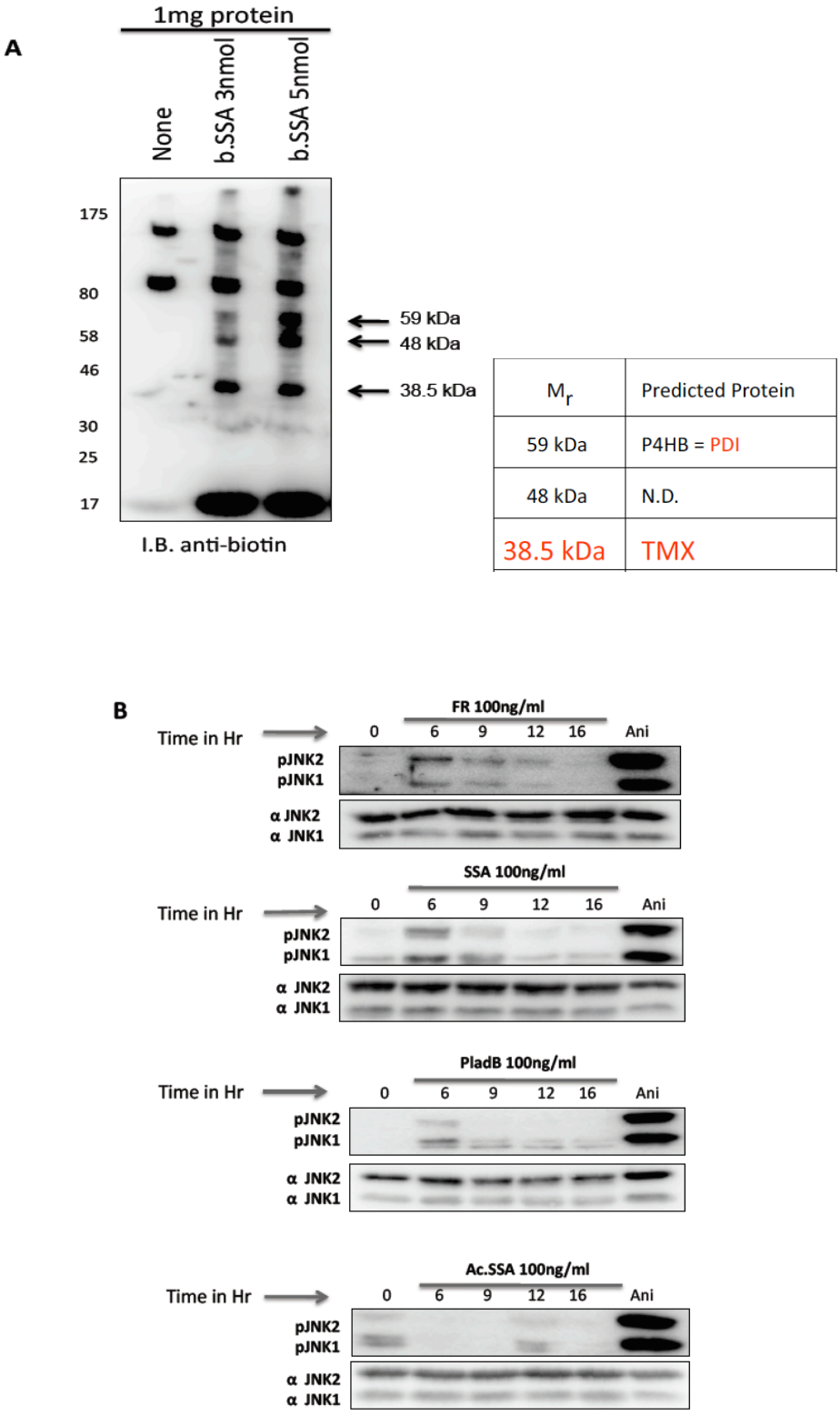


Figure 5.2: *In vitro* binding assay (A) Binding of SSA was confirmed by using biotinylated SSA. Interaction between TMX and SSA was detected by blotting against biotin atibody. (B) Time dependent activation of JNK by SSA, plad B, FR and Ac-SSA.

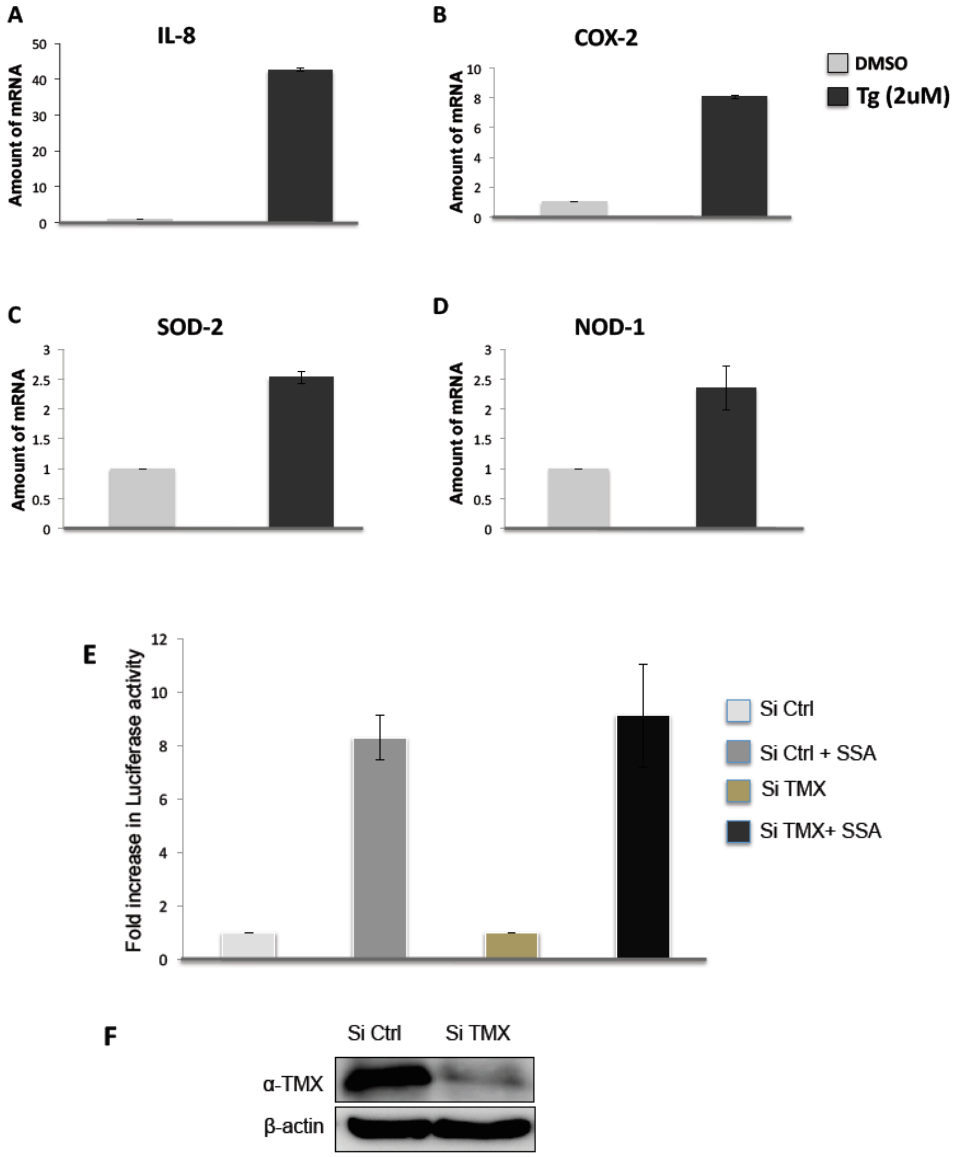


Figure 5.3: Effect of ER stress inducers on gene activation. (A) ER stress inducer, Thapsigargin (Tg) induces NF-kB target genes IL-8, (B) COX-2, (C) SOD-2 and (D) NOD-1. (E-F) Co-transfected A549 cells with TMX siRNA and IL8-Luc construct were resistant to SSA-induced IL-8 gene activation.

Discussion

In unstimulated cells, NF-kB components are in association with IkBs in cytoplasm. The two major and well-studied IkBs proteins in mammals are IκBα and IκBβ. Both can interact with p65 and c-Rel thus indicating that NF-kB activity is carried out by these IkBs isoforms. The IκBα promoter have its own binding sites for IκBα, which means that after degradation by proteasome, it is quickly synthesized to shut down NF-kB response. Thus ensuring that target genes are transiently activated. By today there are different inducers of NF-kB activity. PMA only affect IκBα and thus transiently activates NF-kB target gene while on other hand LPS cause persistent activation of NF-kB pathway by degrading both IκBα and IκBβ (67).

Previously its has been reported that IL-1β increases the mRNA of NF-kB target genes i.e are iNOS, COX-2, VCAM-1 and MnSOD via ERK dependent pathway. ERK inhibitor, PD98059 inhibits iNOS and COX-2 production but fails to inhibit VCAM-1 and MnSOD. In that study author suggested that PD98059 only rescue the degradation of IκBβ but not IκBα. Furthermore, it has been shown that PD98059 inhibit DNA binding ability of p65/50 and p50/50 at 6 h, without having a significant effect at 30 min to 1 h. This suggests that PD98059 does not inhibit the early and transiently activated NF-KB target genes like VCAM-1 and MnSOD in response to IL-1β (68).

Our data suggest that transgene activation primarily requires ERK and NF-kB pathways in response to SSA. We consider four approaches to identify the relative importance of these pathways. First we used transcription factor binding site mutants of IL-8 gene. However, site-directed mutagenesis of transcription factors binding sites gives limited information for transcription factors having binding sites close to each other (69). Secondly, alternative approach was used to knockdown components of NF-kB. In third approach we used an inhibitor of NF-kB pathway, parthenolliod that inhibit SSA–induced IL-8 and CMV promoter activation. The (Fig. 4.2 E) clearly indicated that 40 μM parthenoliod rescue the degration of IκBα but also inhibits ERK

phosphorylation in response to SSA. The last approach to show the importance of ERK pathway; we inhibit ERK phosphorylation by using 100 μM PD98059 and then confirm IL-8 and CMV promoter activity through luciferase assay.

ERK can directly phosphorylate many transcription factors including Ets-1, c-Jun, CREB and c-Myc. ERK can phosphorylate inhibitor κB kinase (IKK) that lead to the activation of the NF-kB transcription factor. Besides this, ERK have more than 160 targets (70). Our data lack the information about specific ERK-mediated transcription factor phosphorylation to enhance gene activation. Further more, we were unable to show a clear crosstalk between ERK and NF-kB pathways. One possibility could be that ERK inhibitor, PD98059 might interfere with the p-65-NF-kB binding to its target DNA. Moreover, Our data lack the affect of SSA upon IκBβ degradation, it might be possible that SSA target IκBβ degradation for persistent activation of CMV and IL-8. In such case we should assume that parthenolide must rescue the degradation of IκBβ. To examine the clear role of ERK cross talk with NF-kB pathway, the best approach would be to over express IκBα and IκBβ and see CMV and IL-8 promoter activity.

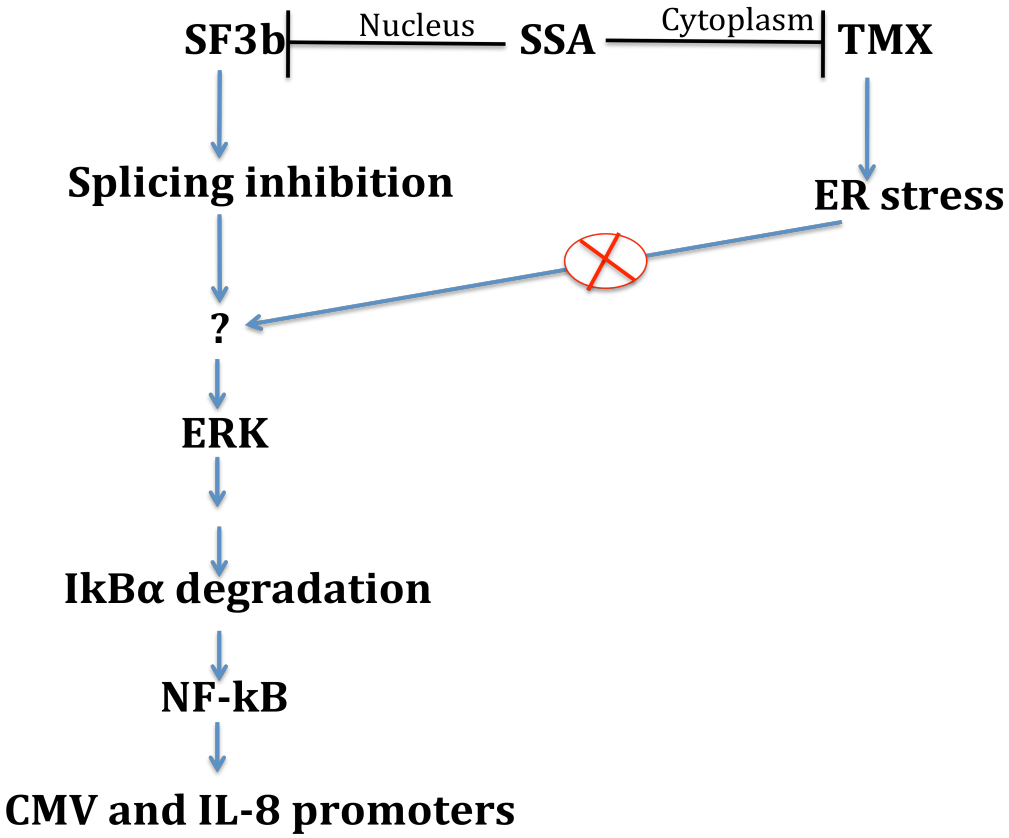
It is well-established fact that SSA regulates the transcription of oncogenes and tumor suppressors (71). The global transcriptome analysis data reveals that SSA down-regulate 24-38%, up-regulate 1-2% and 60-75% gene unchanged upon SSA treatment at 3 h and 12 h respectively (17). I found few genes contains binding sites for NF-kB transcription factor i.e are superoxide dismutase 2, mitochondrial (SOD2), interleukin 8 (IL-8), interleukin 6 (IL-6), v-fos FBJ murine osteosarcoma viral oncogene homolog (FOS) and tumor necrosis factor, alpha-induced protein 3 (TNFAIP3 or A20), which makes only 4-5% of the genes that are up-regulated by SSA. I assume that SSA might target other transcription factors or post-translation modifications to enhance the transcriptional activity of variety of the remaining genes.

It is natural when splicing is inhibited, no more proteins will form and normal cell will eventually die. But why splicing inhibitors only kill cancer cells

selectively? In general malignant cells have higher metabolic rates and have increased splicing rates comparatively to normal cells and are more prone to splice inhibitor or modulators (72). The best strategy is to inhibit the splicing which would eventually inhibit tumor at drug levels that do not affect normal cells. The global transcriptome analysis data showed that many of the genes affected were involved in cell proliferation, anti-apoptosis and angiogenesis (9). I suspect that one possible reason could be that SSA affects many pathways by up and down-regulate gene expression in cancerous cells that might help in killing cancerous cells. Since splicing inhibitors have documented antitumor activity, with some even entering clinical trials, NF-kB activity can be critical, as the pathway generally aids in cell proliferation and survival. Further studies will show whether this induced NF-kB activity aids or counteracts antitumor efficacy.

The IL-8 expression promotes angiogenesis; tumorigenicity and drug induced IL-8 expression confer chemotherapeutic resistance in cancer cells (73). Therefore, suppression of IL-8 production may be a significant therapeutic strategy in targeting the tumor microenvironment.

Overview



SSA binds to SF3b and TMX in the nucleus and cytoplasm respectively. SSA target SF3b to inhibit splicing and TMX to induce ER stress. Splicing inhibition leads to ERK pathway activation by unknown mechanism. It would appear plausible that ERK phosphorylates and activates the upstream kinase of the NF-kB pathway IKK. The end result is the IkBα degradation, allowing NF-kB to translocate to the nucleus to induce CMV and IL-8 genes. The activation of genes is independent of ER stress induced by SSA binding to TMX.

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