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Splicing inhibition induces gene expression through canonical NF-κB pathway and extracellular signal-related kinase activation

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ABSTRACT

Splicing, a process for mRNA maturation, is essential for correct gene expression after transcription. However, recent studies also suggest that splicing affects transcription, but its mechanism remains elusive. We previously reported that treatment with spliceostatin A (SSA), a specific splicing inhibitor targeting the splicing factor SF3b, leads to transcriptional activation of a small subset of genes. To investigate the underlying mechanism we utilized luciferase reporters driven by the Interleukin 8 (IL-8) and cytomegalovirus (CMV) promoters, as both recruit a similar set of transcription factors. We also found that SSA treatment led to increased extracellular signal-regulated protein kinase (ERK) activity and that chemical inhibition of ERK also led to decreased promoter activation. Systematic deletion studies suggested that NF-κB activation is mainly responsible for SSA-induced promoters activation.

1. Introduction

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 [1]. Thus, splicing is a key mechanism for regular gene expression and interference with splicing will affect gene expression. Recently, FR901464 and its methylated derivative spliceostatin A (SSA) (Fig. 1A) were reported for the first time to inhibit pre-mRNA splicing by binding non-covalently to the SF3b sub-complex in the U2 snRNP [2,3]. SSA is chemically more stable than its parent molecule FR901464, which was originally isolated from a *Pseudomonas* fermentation broth. During screening of microbial metabolites, FR901464 was found to activate viral promoters including those of the SV40 and the cytomegalovirus (CMV) [4,5]. Previous studies have shown that SSA leads to transcriptional repression of about 20% of expressed genes, while enhancing transcription of only a small subset [6]. Among the most highly upregulated genes was Interleukin-8 (IL-8). Since IL-8 and CMV promoters are activated by a similar set of transcription factors we 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 [7]. Interleukin-1 (IL-1), tumor necrosis factor alpha (TNF-α) and phorbol 12-myristate 13-acetate (PMA) are well known inducers of IL-8 [7,8]. These inducers can enhance the transcriptional stimulatory activity of viral promoters (SV40 and CMV) as well and are also able to activate signaling kinases [9–12]. Previous investigations suggested that IL-8 production is regulated by extracellular signal-regulated protein kinase (ERK) and transcription factor NF-κB in response to TNFα, IL-1β or PMA [13–16]. 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 we report that SSA treatment induces ERK activation and that SSA treatment leads to enhanced NF-κB activity.

2. Material and methods

2.1. Materials, cell lines and antibodies

A549, NIH3T3, NCL-5 and HeLa cells were maintained in DMEM with 10% FBS and antibiotics. NCL-5 cells are a stably transfected

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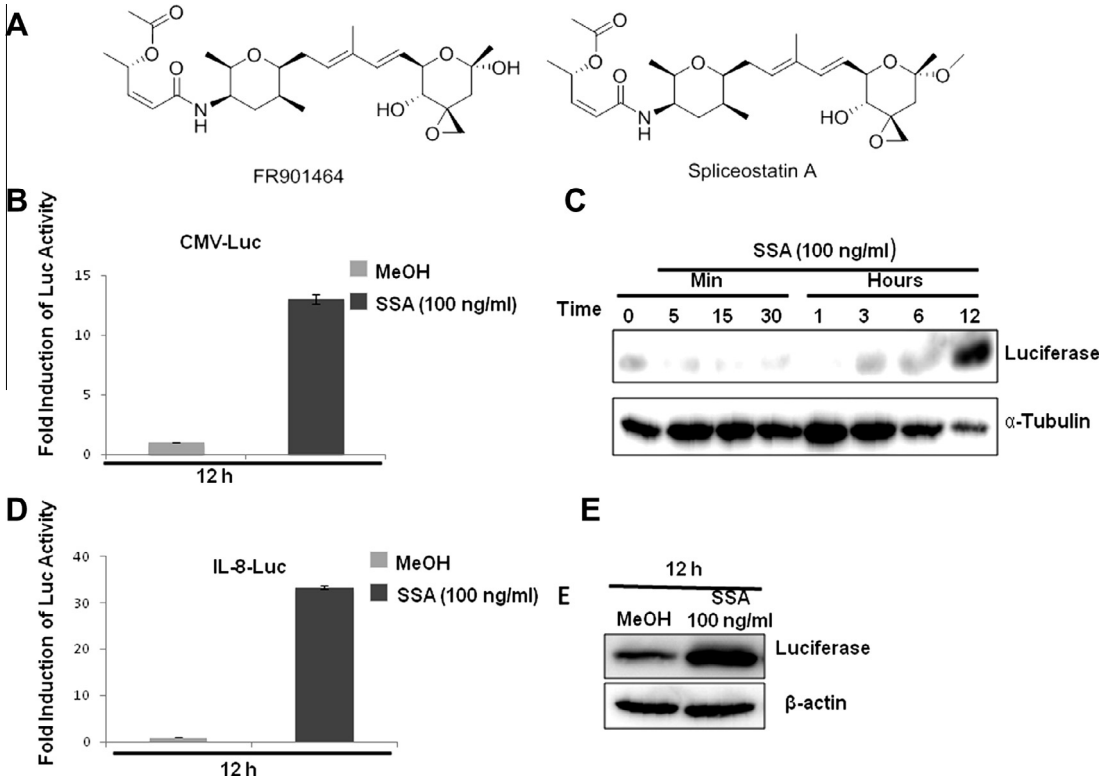

Fig. 1. Spliceostatin A (SSA) treatment leads to CMV and IL-8 promoter activation. Structures of parent compound FR901464 and SSA (A). SSA induced expression of the CMV promoter in NCL-5 cells harboring a CMV-luciferase construct (B). Relative concentration of the luciferase protein by Western blot (C). Similar results were observed with the IL-8 promoter in transiently transfected NIH3T3 cells (D, E).

NIH3T3 cell line with a luciferase reported driven by the CMV promoter [17]. Parthenolide and PD98059 were purchased from Wako Chemicals and Cell signaling technologies, respectively.

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), NF-κB-p65 (sc-109) and IκBα (sc-371) were purchased from Santa Cruz Biotechnology. Antibodies against SAP130, SAP155 and β-actin were purchased from Abcam. A mouse monoclonal anti α-Tubulin (B-5-1-2) antibody was from Sigma. Cells were lysed in NETN lysis buffer (50 mM tris–Hcl pH7.5, 150 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40), containing 1× protease inhibitor cocktail (Roche), and protein concentrations were determined by Bradford assay. Cell lysate was dissolved in 1× SDS–PAGE sample buffer and heated to 95 °C for 5 min. 20 μg of total protein was 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 were detected with Immobilon™ Western Chemiluminescent HRP substrate (Millipore) in a LAS-3000 image analyzer (GE Healthcare).

2.3. Immunofluorescence microscopy

Cells grown on a 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 the anti-NF-κB-p65 primary antibody (1:200 dilution), for 1–2 h followed by an Alexa Fluor 594-conjugated secondary antibody (Invitrogen) for 20 min. Slides were mounted with mounting medium (Vector Laboratories), and images were taken using a Delta Vision fluorescent microscope (SEKI Technotron Corp.).

2.4. Expression and knockdown

NIH3T3 and A549 cells were seeded at 5×10^4 cells per well in a 24 well multi-well plate and transfected with expression vectors using Lipofectamine™ LTX (Invitrogen) for 48 h or FuGENE®+ HD (Roche) for 72 h. The pCR3.1-Luc vector containing the wild type CMV promoter was kindly provided by Dr. Weber [18]. Deletion mutants and transcription factor point mutants of IL-8 were prepared as described [19]. Cells were lysed in NETN buffer after treatment with compounds at the indicated time points and luciferase activity was measured with a SpectraMax Luminometer (Molecular Devices). Small Interference RNA (siRNA) for SAP130, SAP145, SAP155 and control non-specific siRNA sequences were from Dharmacon, while those for NF-kB-p65 were from Nippon Gene (Table-S1).

3. Results

3.1. Effect of spliceostatin A (SSA) on IL-8 and CMV promoters

Previous studies suggest that SSA enhances the transcription from the SV40 viral promoter in M-8 cells [2] and the CMV promoter in NCL-5 cells [5]. A recent report on microarray data of HeLa cells treated with SSA showed significant upregulation of the IL-8 gene. To reproduce previous findings we began our study with luciferase reporter assays based on the CMV and IL-8 promoters. (Fig. 1B–E). SSA-induced gene activation proceeds in a

time-dependent manner (Fig. S1A). These results show that SSA enhances the activity of IL-8 and CMV promoters. Acetylated SSA, an inactive derivative of SSA as a control, showed no effect (data not shown). Hence, increased expression appears due to splicing inhibition and does not stem from off-target activity. To further confirm that splicing inhibition caused the observed activity, we utilized RNA interference against subunits of the SF3b complex (Fig. S1B). Since knockdown of spliceosomal components alone also led to promoter activation, we can rule out an alternative cause for SSA's effect on transcription (S1C, D).

3.2. The ERK MAPK pathway plays a role in IL-8 and CMV promoter up-regulation in response to SSA

It is well documented that MAP kinase signaling significantly up-regulates IL-8 gene expression [7]. To elucidate the role of MAPK signaling pathways on IL-8 and CMV promoter stimulation, we monitored kinase activation by Western blot analysis. We found that SSA caused ERK activation in a time-dependent manner. A significant increase in phosphorylation was observed starting three hours past SSA challenge (Fig. 2A). Furthermore, the activation of IL-8 and CMV promoters was sensitive to PD90859, a MEK inhibitor (Fig. 2B–D). We found that the induction of CMV promoter-driven transcription was inhibited upon addition of PD98059 in a dose-dependent manner (Fig. S2). These results indicate a connection between ERK activity and activation of the CMV and IL-8 promoters.

3.3. An essential role for NF-κB in the regulation of IL-8 and CMV promoters

The general architecture of the IL-8 promoter with its transcription factor binding sites is well established (Fig. 3A). We utilized promoter deletion mutants as shown to detect which region was involved in SSA-dependent transactivation. All constructs could be activated to similar extent except for the shortest construct, which only contained 50 nucleotides upstream of the transcription start site. The next longer construct containing 98 upstream nucleotides showed already significant activation upon SSA treatment. Therefore, the key regulator most likely resides between nucleotides 50 and 98, which best corresponds to both a known NF-κB binding site as well as the binding sequence for NF-IL6 (Fig. 3B). To identify the transcription factor responsible, we utilized variants of a construct containing 133 upstream nucleotides, with each of the known transcription factor binding sites individually deleted (Fig. 3C). Deletion of the NF-κB site abolished transactivation almost completely, while rendering the NF-IL6 binding region nonfunctional had, if anything, a stimulatory effect (Fig. 3D).

3.4. SSA affects the canonical NF-κB pathway

NF-κB activation takes place following the phosphorylation of IκBα, which results in its ubiquitination and rapid degradation by the proteasome, allowing NF-κB to translocate to the nucleus [14]. Otherwise the IκB–NF-κB complex will remain in the cytoplasm [20]. Using immuno-histochemistry, we observed the cellular localization of the NF-κB subunit p65 under SSA treatment. As shown in Fig. 4A, the amount of nuclear p65 increased after SSA application. Meanwhile, IκBα was decreased in a time-dependent manner (Fig. 4B). Therefore, it appears most likely that lack of sufficient IκBα leads to persistent activation of the NF-κB pathway. Furthermore, we found that parthenolide, a NF-κB pathway inhibitor that specifically inhibits the phosphorylation of IκBα by upstream kinases [21], also decreased the stimulatory effect of SSA upon CMV and IL-8 promoters (Fig. 4C and D).

As further confirmation we knocked down the p65 subunit to see if NF-κB is required for transcriptional activation upon SSA treatment (Fig. S3). Knockdown of p65 clearly inhibited the SSA-induced IL-8 gene transcription activity. Furthermore, cells treated with parthenolide showed reduced levels of IκBα, however the NF-κB inhibitor did appear to rescue the remaining protein from degradation upon SSA treatment (Fig. S4).

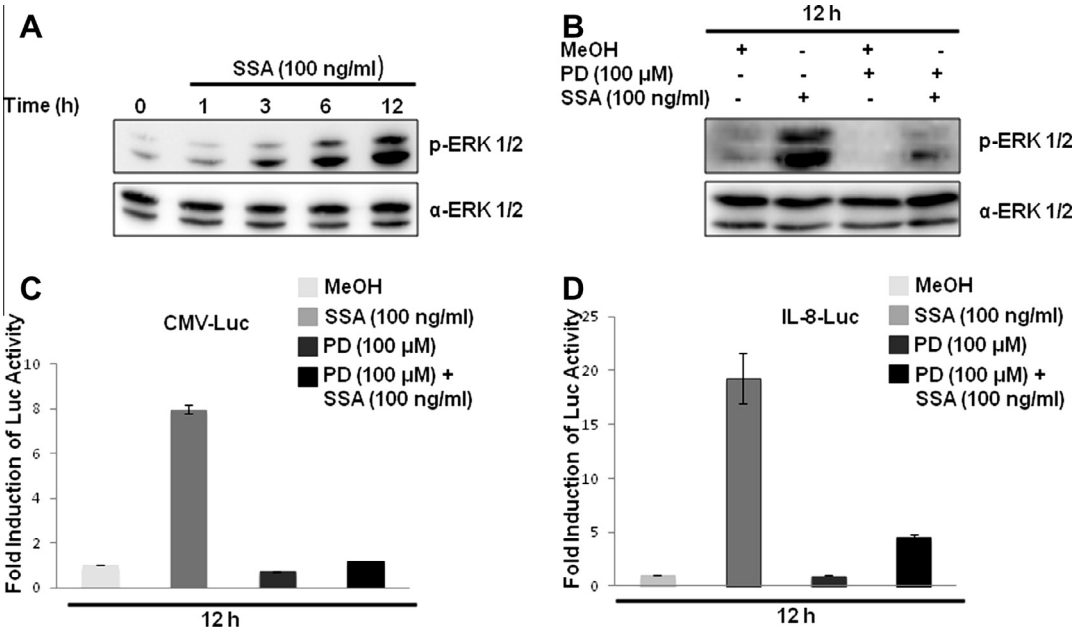

Fig. 2. Activation of the ERK signaling pathway. SSA (100 ng/ml) treatment led to increased ERK activation over time in NCL-5 cells as determined by Western blot (A). NCL-5 cells were pretreated with MEK inhibitor PD098059 (100 μM) for 1 h followed by SSA (100 ng/ml) for 12 h. PD reduced SSA-induced ERK phosphorylation (B) and CMV promoter activation confirmed by luciferase assay (C). Similar results were observed with the IL-8 promoter in transiently transfected A549 cells, confirmed by luciferase assay (D). Error bars represent standard error of mean.

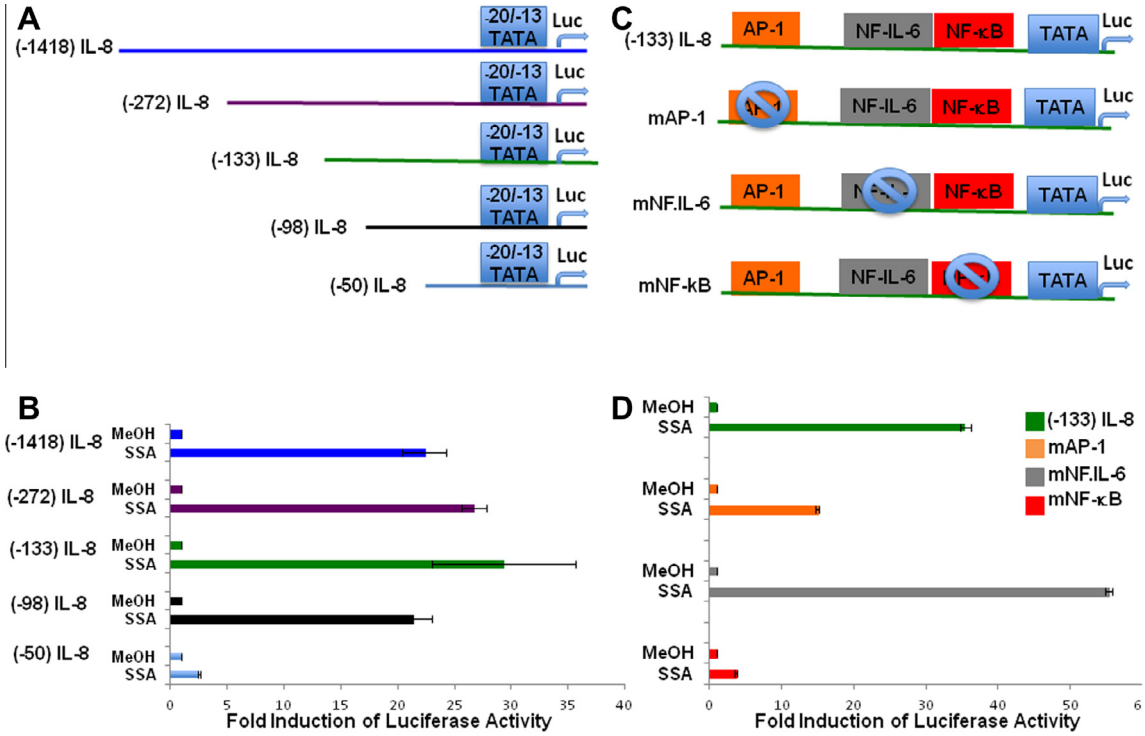

Fig. 3. Identification of the transcription factor responsive to SSA treatment. Constructs containing various lengths of the IL-8 promoter linked to a luciferase reporter were used (A). NIH3T3 cells were transiently transfected with 5′ flanking deletion mutants of the IL-8 gene for 48 h, followed by incubation with and without SSA (100 ng/ml) for 12 h. Activity of each mutant was measured by luciferase assay (B). Diagram outlining deletion mutants of the IL-8 core promoter, lacking individual transcription factor binding sites (C). NIH3T3 cells were transiently transfected with wild type, IL-8 (-133)-Luc, mAP-1, mNF-IL6 and mNF-kB deletion mutants. Luciferase activity 48 h post transfection was measured upon 12 h SSA (100 ng/ml) treatment. Constructs without the NF-κB binding site become unresponsive to SSA-induced promoter activation.

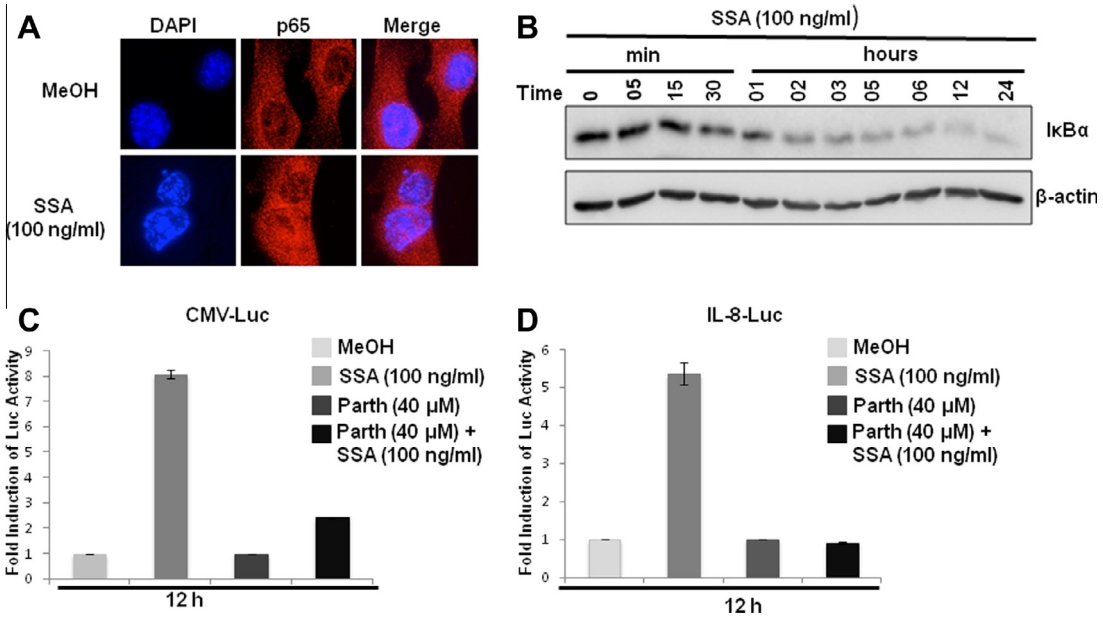

Fig. 4. SSA treatment leads to NF-κB activation. p65 translocated to the nucleus upon SSA treatment. NIH3T3 cells were treated for 12 h with either methanol (upper panel) or SSA (100 ng/ml) (lower panel), nuclear localization of the p65 subunit was determined by immunostaining (A). IκBα degradation was observed by Western blot at different time intervals (B). Levels of CMV (C) and IL-8 (D) promoter activation by SSA (100 ng/ml) for 12 h were determined by luciferase reporter assay in transiently transfected A549 cells in the presence or absence of 40 μM parthenolide.

4. Discussion

Here we show that part of the transcriptional upregulation observed during splicing inhibition, stems from activation of the NF-κB signaling pathway. The promoter region of IL-8 contains putative binding sites for NF-κB, NF-IL6 and AP-1 [22]. Site directed mutagenesis of each binding site showed that SSA mediated transactivation mainly depends on NF-κB. This was consistent with results obtained via small interfering RNA (siRNA) silencing of the p65 subunit of NF-κB or use of the NF-κB-specific inhibitor

parthenolide. This study presents evidence that the specific activation of a small subset of genes during splicing inhibition depends on ERK activity and on NF-κB.

As the amount of the key inhibitory factor, IκBα decreases with SSA treatment, more NF-κB can translocate to the nucleus. Indeed, other NF-κB target genes, such as superoxide dismutase (SOD2), experience upregulation under SSA treatment [6]. Furthermore, we found evidence for an involvement of the ERK kinase pathways in gene activation during splicing inhibition. It would appear plausible that ERK phosphorylates and activates the upstream kinase of the NF-κB pathway IKK [23]. Our data are consistent with a connection between ERK signaling and NF-κB activity [24].

In summary, we have documented that chemical inhibition of splicing activated ERK signaling and the transcription factor NF-κB. This provides the first explanation how inhibition of pre-mRNA splicing can have an activating effect on a specific subset of genes. Since splicing inhibitors have documented antitumor activity, with some even entering clinical trials, NF-κB activity can be critical, as the pathway generally aids in cell proliferation and survival. Further studies will show whether this induced NF-κB activity aids or counteracts antitumor efficacy.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2014.02.018.

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