Inhibition of splicing and nuclear retention of pre-mRNA by spliceostatin A in fission yeast

Chor-Wai Lo a,b, Daisuke Kaida a, Shinichi Nishimura a, Akihisa Matsuyama a, Yoko Yashiroda a, Hiroshi Taoka a, Ken Ishigami c, Hidenori Watanabe c, Hidenori Nakajima d, Tokio Tani c, Sueharu Horinouchi b, Minoru Yoshida a,f,*

a Chemical Genetics Laboratory, RIKEN, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan
b Department of Biotechnology, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan
c Department of Applied Biological Chemistry, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan
d Drug Discovery Research, Fermentation Research Laboratories, Astellas Pharma Inc., 5-2-3 Tokodai, Tsukuba, Ibaraki 300-2698, Japan
e Department of Biological Sciences, Graduate School of Science and Technology, Kumamoto University, 2-39-1 Kurokami, Kumamoto 860-8555, Japan
f Japan Science and Technology Corporation (JST), CREST Research Project, Kawaguchi, Saitama 332-0012, Japan

Received 4 October 2007
Available online 15 October 2007

Abstract

Nuclear retention of pre-mRNAs is tightly regulated by several security mechanisms that prevent pre-mRNA export into the cytoplasm. Recently, spliceostatin A, a methylated derivative of a potent antitumor microbial metabolite FR901464, was found to cause pre-mRNA accumulation and translation in mammalian cells. Here we report that spliceostatin A also inhibits splicing and nuclear retention of pre-mRNA in a fission yeast strain that lacks the multidrug resistance protein Pmd1. As observed in mammalian cells, spliceostatin A is bound to components of the SF3b complex in the spliceosome. Furthermore, overexpression of nup211, a homolog of Saccharomyces cerevisiae MLP1, suppresses translation of pre-mRNAs accumulated by spliceostatin A. These results suggest that the SF3b complex has a conserved role in pre-mRNA retention, which is independent of the Mlp1 function.

Keywords: Spliceostatin A; Fission yeast; Pre-mRNA; SF3b; Splicing; Pre-mRNA retention

Most primary eukaryotic transcripts (pre-mRNAs) are interrupted by non-coding sequences (introns) that must be removed, and coding sequences (exons) are joined together to yield mature mRNAs before export from the nucleus for translation [1]. Translation of pre-mRNAs and partially spliced mRNAs will lead to the production of proteins containing intron-derived sequences, which is potentially harmful. Several proteins have been shown to function to avoid export and translation of pre-mRNAs. For example, Mlp1, a budding yeast protein that localizes at the nuclear pore complex, prevents pre-mRNAs export into the cytoplasm without affecting splicing [2]. On the other hand, a newly identified pre-mRNA RETention and Splicing (RES) complex containing Snu17, Bud13, and Pm1 is required for efficient splicing as well as nuclear retention of pre-mRNA [3]. In the nucleus, Rrp6, an exosome component, degrades mRNAs with an abnormal poly (A) tail [4]. However, it is unclear whether these “security mechanisms” in budding yeast are conserved in other eukaryotes.

Recently, we reported that antitumor substances, FR901464 and its methyl ketal derivative spliceostatin A (SSA) (Fig. 1), inhibit splicing and allow pre-mRNA translation [5]. FR901464 was originally isolated from the fermentation broth of a bacterium Pseudomonas sp. as an
antitumor compound that enhances the transcription from the SV40 promoter and causes cell cycle arrest at the G1 and G2/M phases [6,7]. SSA is a chemically stable derivative of FR901464 [5,8]. In the course of our screening for the target protein, SSA was found to bind to the SF3b complex, a sub-complex of U2 snRNP in mammalian cells. Furthermore, pre-mRNA is accumulated and translated in SSA-treated cells, suggesting that SSA inhibits splicing and nuclear retention of pre-mRNA by binding to the splicing complex, SF3b, although the precise mechanism remains to be elucidated [5].

In this study, using the SSA-sensitive Schizosaccharomyces pombe strain, we show that SSA causes accumulation and translation of pre-mRNA by binding to the SF3b complex. Furthermore, ts mutants of SF3b components exhibit similar phenotypes to SSA-treated cells at the non-permissive temperature. These observations demonstrate that the SF3b is a component of the mRNA security mechanism conserved from yeast to mammal.

Materials and methods

See Supplementary Data.

Results

**Pmd1 is responsible for spliceostatin A resistance in S. pombe**

We wanted to analyze the effect of SSA on pre-mRNA splicing in S. pombe. S. pombe was resistant to SSA at least up to 12.5 μg/ml. Some of the drug efflux pumps in *S. pombe* might be responsible for the intrinsic resistance to SSA. The *S. pombe* genome contains 11 ATP-binding cassette (ABC) transporters that are potentially involved in SSA resistance. Therefore, we deleted all the genes encoding ABC transporters by homologous recombination, and tested for their drug sensitivity. Among 11 disruptants, 10 strains still showed resistance to SSA. However, the *pmd1* disruptant was highly sensitive to SSA (Table 1). Thus, we conclude that Pmd1 is responsible for the SSA resistance in wild-type *S. pombe*, and used the Δ*pmd1* strain for further studies.

**Spliceostatin A induces pre-mRNA accumulation and translation in S. pombe**

To examine if SSA inhibits splicing in fission yeast, we extracted the total RNA from SSA-treated Δ*pmd1* cells and analyzed the transcript of *tdf1* that contains introns by reverse transcriptase (RT)-PCR (Fig. 2A). As shown in Fig. 2B, the *tdf1* pre-mRNAs was accumulated in SSA-treated cells. Similar pre-mRNA accumulation was observed with another intron containing gene *cct8* (data not shown). The unspliced mRNA accumulation was accompanied by a marked decrease in the mature mRNA level, indicating that SSA inhibits *in vivo* splicing in fission

<table>
<thead>
<tr>
<th>Strain</th>
<th>MIC of SSA (μg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>&gt;12.5</td>
</tr>
<tr>
<td>Δ<em>SPAC15A10.01</em></td>
<td>&gt;12.5</td>
</tr>
<tr>
<td>Δ<em>of1</em></td>
<td>&gt;12.5</td>
</tr>
<tr>
<td>Δ<em>SPAC30.04c</em></td>
<td>&gt;12.5</td>
</tr>
<tr>
<td>Δ<em>anm1</em></td>
<td>&gt;12.5</td>
</tr>
<tr>
<td>Δ<em>pmd1</em></td>
<td>0.78</td>
</tr>
<tr>
<td>Δ<em>SPBC359.05</em></td>
<td>&gt;12.5</td>
</tr>
<tr>
<td>Δ<em>ahc1</em></td>
<td>&gt;12.5</td>
</tr>
<tr>
<td>Δ<em>SPAC3F10.11c</em></td>
<td>&gt;12.5</td>
</tr>
<tr>
<td>Δ<em>SPAPB24D3.09c</em></td>
<td>&gt;12.5</td>
</tr>
<tr>
<td>Δ<em>mnt1</em></td>
<td>&gt;12.5</td>
</tr>
<tr>
<td>Δ<em>SPBC9B6.09c</em></td>
<td>&gt;12.5</td>
</tr>
</tbody>
</table>
Next, we tested whether the accumulated pre-mRNAs can be translated into proteins. To this end, we designed a new reporter system named pIntron-Z based on the cct8 gene, which enables one to measure the level of translation from pre-mRNA (Fig. 2C). β-Galactosidase cannot be produced from normally spliced mRNA due to the stop codon. If pre-mRNA is translated, however, functional β-galactosidase will be generated, because frameshift allows read-through. The β-galactosidase activity was almost undetectable in untreated cells, in which pIntron-Z was integrated. In contrast, a significant level of β-galactosidase activity emerged in the cells treated with SSA (Fig. 2D), demonstrating that SSA treatment caused translation of intron-containing mRNAs in S. pombe.

**Spliceostatin A binds to the SF3b splicing factor complex**

We have previously shown that SSA binds to SF3b [5], one of the sub-complexes in the spliceosomal U2 snRNP in mammalian cells [1]. The ability of SSA to bind to SF3b components in S. pombe was analyzed by pull-down assay using S. pombe strains expressing GFP-tagged Prp10 and Prp12, homologs of SAP155 and SAP130, respectively [9,10]. As shown in Fig. 3A, biotinylated-SSA (bio-SSA) bound to both GFP-tagged Prp10 and Prp12 in the cell extracts. This binding was blocked by an excess amount of SSA added to the cell extracts (Fig. 3B). On the other hand, Ac-SSA, an inactive form of SSA [5], did not compete with the bio-SSA...
binding to Prp10 or Prp12 (Fig. 3B). Previous studies showed that SSA-binding to SF3b was reversible in mammalian cells [5]. Indeed, the proteins were removed from bio-SSA by washing an SDS-containing buffer (Fig. 3C), suggesting that SSA specifically binds to the SF3b complex in a non-covalent manner also in S. pombe.

Mutations in prp10 or prp12 lead to translation of intron-containing mRNA

To elucidate whether targeting of SF3b by SSA causes pre-mRNA translation in fission yeast, we used pIntron-Z for detecting pre-mRNA translation in two temperature-sensitive (ts) mutants, prp10-1 and prp12-1, which are defective in pre-mRNA splicing [9,10]. RT-PCR analysis showed that tdf1 mRNA was correctly spliced in the prp10-1 or prp12-1 mutants at 26°C (Fig. 4A). In contrast, a large amount of pre-mRNA was accumulated in the mutant cells incubated for 3 h after the temperature shift-up to 37°C (Fig. 4A), as described previously [9,10]. Almost no increase in the β-galactosidase activity was detected in the mutants at the permissive temperature (26°C). However, a relatively high level of β-galactosidase activity was detected in the mutant cells cultured at 37°C, although the levels were lower than in wild-type cells treated with SSA (Fig. 4B). These results suggest that the SF3b complex is involved in a mechanism preventing pre-mRNA translation in S. pombe.

Discussion

In this study, we found that SSA binds to SF3b and inhibits pre-mRNA splicing in the fission yeast S. pombe using the pmd1 disruptant. pmd1 was originally identified as a multi-drug resistance gene related to mammalian Mdr1 [11]. The present result indicates that SSA is a sub-

Fig. 4. Pre-mRNA translation level in various cells. (A) RT-PCR analysis of tdf1 mRNA. MKPUC cells were cultured in YE medium at 26 or 37°C with or without SSA for 3 h. Prp10 or prp12 ts mutants were cultured in YE medium at 26°C to the mid-log phase, and cultured for 3 h at 37°C. RT-PCR was performed as described in Fig. 2B. Pre-mRNA and mature mRNA are indicated as P and M, respectively. As a loading control, exon 4 of cct8 was amplified by RT-PCR. (B) Pre-mRNA translation analysis. MKPUC and prp mutant cells transformed with the reporter system were cultured for 3 h at 37°C. β-Galactosidase activity in the cell extracts was measured. (C) Nup211 prevents pre-mRNA translation. Strains with integrated ORF-FFH fusion vectors were grown for 16 h in minimal medium (MM) in the presence or absence of thiamine. After induction or repression of FFH-tagged proteins, 5 µg/ml of SSA was added and cells were incubated for 3 h. β-Galactosidase activity of the cell extract was measured. The error bars represent ±SD.
strate for Pmd1. We also showed that fission yeast SF3b contains the SSA-binding pocket and plays an important role in nuclear retention of pre-mRNA in fission yeast. Several security mechanisms to avoid pre-mRNA translation have been proposed in budding yeast *S. cerevisiae* [2,3]. In this report, overexpression of *nup211*, a homolog of *S. cerevisiae MLP1*, suppressed translation of pre-mRNAs caused by SSA treatment. Nup211 localizes at the nuclear periphery [12]. Therefore, it is likely that Nup211 is a functional homolog of Mlp1 acting as a gatekeeper to inhibit the pre-mRNA export. However, we could not carry out experiments using the *nup211* disruptant, because *nup211* is an essential gene unlike *MLP1* [2,12]. Nup211, Mlp1, and mammalian TPR have a highly homologous region among these three proteins [12,13]. Because it was also reported that TPR is required for mRNA export and Mlp1 is not [13,14], it is intriguing to elucidate how these proteins discriminate mature mRNAs from pre-mRNAs during the nuclear export.

Since *nup211*-overexpression can rescue the defect of pre-mRNA retention by inhibiting the SF3b complex in SSA treated cells (Fig. 4C), these two mechanisms seem to function in parallel for pre-mRNA retention. This hypothesis is also supported by the notion that the intact 5′ splicing site is required for the nuclear retention by Mlp1 [2] and that SF3b binds to the branch point sequence [15]. Other proteins concerning pre-mRNA security mechanisms in budding yeast are components of the RES complex [3]. *S. pombe* Cwf26 and Cwf29 have high similarity to budding yeast Bud13 and Ist3, respectively [16], although a homolog of Pml1 is not found in *S. pombe*. However, overexpression of *cwf26* and *cwf29* cannot suppress the pre-mRNA translation by SSA treatment (Fig. 4C). Although it is still unclear whether the fission yeast has the functional RES complex, it is possible that Cwf26 and Cwf29 function as a security mechanism but co-overexpression of these two genes is required for suppression of pre-mRNA translation. Further studies are needed for elucidating the functional roles of these proteins and their relationships to SF3b.

**Acknowledgments**

We are grateful to the RIKEN Brain Science Institute’s Research Resources Center for DNA sequencing analysis. This work was supported in part by the CREST Research Project, the Japan Science and Technology Agency, The Strategic Research Programs for R&D, RIKEN, and a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

**Appendix A. Supplementary data**

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2007.10.029.

**References**


