論文の内容の要旨

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論文題目

Studies on the mode of action of FR901464 that inhibits pre-mRNA processing in fission yeast.

(分裂酵母の mRNA プロセシングを阻害する FR901464 の作用機序に関する研究)

Introduction

RNA splicing is one of the major steps in the control of gene expression in eukaryotes. Genes are often interrupted by intervening sequences (IVSs or introns) that must be removed by a two-step transesterification mechanism, and the functional sequences (exons) are joined together before they are translated into proteins. A defect in splicing of certain introns results in production of aberrant proteins. In the cells, some molecular mechanisms have been identified to be responsible for preventing pre-mRNAs from being exported to the cytoplasm before they are correctly processed. For example, the branchpoint splicing factor, BBP/SF1, and the newly identified RES complex have been shown to be required for pre-mRNA retention in the nucleus. Most recently, Mlp1, a protein localized at the nuclear periphery, plays an important role in preventing pre-mRNAs from being exported to the cytoplasm. To date, there are several lines of evidence for the link between these error-induced mRNAs and human diseases. For example, retinitis pigmentosa (RP), a retinal degeneration that causes blindness, is due to mutations in the autosomal dominant RP (adRP) genes. However, the molecular mechanisms underlying the pathogenesis of RP are not well understood. Many RP genes are expressed predominantly in retina, and four nonretina-specific adRP genes have been shown to encode pre-mRNA splicing factors involved in the spliceosome assembly. It has been reported that some mutations in several RP genes are responsible for RNA processing defects and retinal degeneration. Thus elucidation of the mechanism of mRNA

processing will help find new therapeutic strategies for genetic disease treatments and for a better understanding of genetic information and gene expression.

Classical genetics, which starts from the identification of particular mutations, is one of the best approaches for understanding the cellular function of a protein. In recent years, chemical genetics involving the use of small molecules that inhibit the function of proteins has been widely used for unveiling the biological systems forming complicated networks.

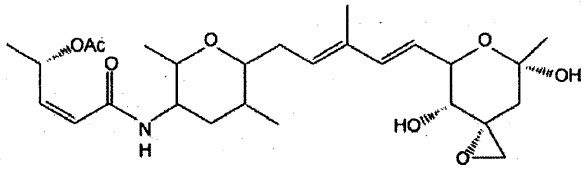

Fig.1 **FR901464**

FR901464 (FR) is a novel antitumor substance, which promotes the transcription of the SV40 early gene. FR has also been shown to induce arrest in G1 and G2/M phases in the cell cycle and internucleosomal degradation of genomic DNA with the same kinetics as for activation of the SV40 promoter-dependent cellular transcription in the reporter-introduced MCF7 (M-8) cells. In this laboratory, FR induced accumulation of a C-terminal truncated form of one of the CDK inhibitors, p27. Extension studies have shown that the short form of p27 was intron-containing mRNA transcripts. However, the mechanism by which FR induces translation of the unspliced mRNAs is yet to be elucidated. In this study, fission yeast *Schizosaccharomyces pombe* was used as a genetic model system to identify the molecular target of FR.

Biotinylated-FR binding proteins

For determination of the mode of action of FR that inhibits pre-mRNA processing, a biotinylated FR (bio-FR) derivative was synthesized as an affinity probe for identification of cellular FR-binding proteins. Prp10, a spliceosome-associated factor in the SF3b complex, was isolated to be the FR binding protein by LC-MS/MS. Studies have shown that the SF3b complex in the mammalian cells contains SAP49/SF3b50, SAP130/SF3b130, SAP145/SF3b145, SAP155/SF3b155, and two other small proteins, SF3b14b and SF3b10. The homologues corresponding to these subunits are identified in *S. pombe* as Sap49, Prp12, Sap145, Prp10, Ini1, and SPBC211.05, respectively. These proteins associate with one another in purified U2 snRNP in the nuclear extracts and play an important role in excision of introns from pre-mRNA.

The ability of FR to bind to other SF3b components was analyzed by pull-down assay using

green fluorescent protein (GFP)-tagged Prp12 and Sap145. As a result, bio-FR also bound to GFP-tagged Prp12. Furthermore, a stronger GFP signal was detected when the binding assay was performed in high salt condition (300 mM NaCl). A previous study reported that the yeast SF3b complex was released from the larger U2snRNP complex when the KCl concentration was increased from 150 mM to 500 mM. In this study, an increase in the salt concentration may release the SF3b complex from U2snRNP, thereby allowing FR to bind to the target proteins. However, the binding of bio-FR to GFP-tagged Sap145 was yet to be detected. In competition-binding assay, the binding was completely blocked when the active FR was added in the assay 1 h before the addition of bio-FR, indicating that the active FR competed with bio-FR for binding to target proteins. Similar experiments showed that inactive FR did not compete with bio-FR, suggesting that bio-FR specifically binds to Prp10 and Prp12. In addition, the association was unstable in the presence of SDS, indicating that the interaction may be mediated by non-covalent binding to FR.

FR901464 induced pre-mRNA accumulation and translation in fission yeast.

Effects of the FR binding to SF3b on *in vivo* splicing in *S. pombe* were examined by using reverse transcriptase-PCR. Because the wild-type cells shows resistance to FR, *pmd1*, a gene encoding an ATP-binding cassette (ABC) transporter, was disrupted. The mutants were viable and exhibited hypersensitivity to FR. RT-PCR analysis on the TFIID gene containing three introns showed accumulation of the TFIID pre-mRNA in the presence of FR. The occurrence of unspliced mRNA was accompanied by a strong decrease in the mature mRNA level.

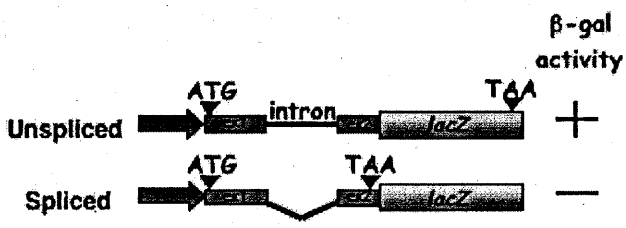

Fig. 2 A reporter system was used to detect pre-mRNA translation.

If the accumulated pre-mRNAs are allowed to be translated, the proteins containing polypeptides derived from the intron sequences should be generated. For detection of proteins containing intron-derived sequences, a reporter was designed (Fig. 2), in which only unspliced mRNA could be translated into the functional enzyme, β-galactosidase. This reporter was introduced into the *pmd1Δ* mutant strain. The β–galactosidase assay indicated that unspliced mRNAs were exported and translated into the enzyme in FR-treated cells.

Abnormal nuclear morphology induced by FR901464.

FR also induced abnormal nuclear morphology in fission yeast. The result of DAPI-staining 3 h after the FR challenge showed that the nucleus was deformed in a way that the nucleolar region was expanded. For further investigation of the mechanism underlying the abnormal nuclear structure, the localization of several nucleolar proteins tagged with YFP was observed. Some of the YFP-tagged proteins appeared to have increased in the intensity of the fluorescence after treatment with FR, thereby causing the abnormal nucleolar structure. In contrast, cellular distribution of proteins that are normally localized in the nuclear envelope was not changed.

Pre-mRNA processing mutants

Prp10 and *prp12* of the fission yeast encode the essential proteins highly homologous with human splicing factors. Temperature-sensitive (ts) mutations of *prp10* and *prp12* cause defects in pre-mRNA splicing at the nonpermissive temperature. In this study, FR was found to bind to Prp10 and Prp12 in the bio-FR binding assay. Therefore, it seems likely that the mutations allow translation of the pre-mRNAs. In support of this hypothesis, the two *prp* mutants were transformed with the reporter and β-galactosidase activity was assayed after a 3-h incubation at the restrictive temperature. As expected, the β-galactosidase activity at a relatively high level was detected in the mutants, indicating that unspliced mRNAs accumulated in the cells were translated into the proteins. Several other *prp* ts mutants transformed with the reporter also exhibited β-galactosidase activity after shifting to the nonpermissive temperature.

Conclusions

1. Splicing factor SF3b was identified as the FR target in this study.
2. Temperature-sensitive mutations of *prp10* and *prp12* caused accumulation and translation of pre-mRNAs.
3. Aberrant nuclear structure was observed in FR-treated cells, accompanied by the expanding of nucleolar region that might cause the abnormality in the nuclear structure.