Identification of novel interactor of evolutionarily conserved splicing factor Snu66

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Abstract

Pre-mRNA splicing is a crucial step of gene expression and involves the removal of protein-noncoding intronic sequences. The process is catalyzed by the spliceosome, which is a multi-megadalton ribonucleoprotein (RNP) complex comprising both protein and RNA components. The spliceosome is assembled on pre-mRNA through several steps involving in sequential interactions of five small nuclear RNPs and hundreds of splicing factors, and is catalytically activated prior to splicing reaction. Snu66 is an evolutionarily conserved protein and functions during the transition step of the spliceosome activation via a yet unknown mechanism. Here, we identified by yeast two-hybrid screen a putative AAA+ ATPase as a novel interactor of Snu66, and mapped the interaction regions in both sides. The interaction is most likely biologically relevant since the interacting domains are highly conserved among eukaryotes. Importantly, null allele of gene encoding the ATPase exhibited a synthetic growth phenotype with splicing mutants, indicating its important role in splicing. Overall, our results suggest that the enzyme as a novel potential mediator in the complex event of spliceosome activation.

Introduction

Splicing of eukaryotic nuclear pre-mRNA happens in a huge ribonucleoprotein (RNP) complex, the spliceosome. This complex comprises five small nuclear RNA (snRNAs), namely U1, U2, U4, U5 and U6, and more than 300 associated protein components[1–3]. Unlike the ribosome, a ribonucleoprotein of comparable complexity and size, the spliceosome is highly dynamic and must be *de novo* assembled on each intron prior to splicing reaction[1,2]. Assembly of the spliceosome takes place in a step-wise manner (Figure 1)[1,2]. First, U1 and U2 snRNPs recognize the 5'-splice site and branch-point region of the pre-mRNA, respectively, to form a prespliceosome (complex A). Next, complex B or precatalytic spliceosome is assembled by the association of preformed U4.U6/U5 tri-snRNPs with the complex A. Subsequently, after several changes in conformation and composition and help of eight members of RNA helicase family the spliceosome becomes activated and be able to catalyze the splicing reaction[2].

During the transition between precatalytic and activated spliceosomes, continuous change in spliceosomal components involving a number of proteins occurs. Snu66, a highly conserved splicing factor among eukaryotes, associates with the preformed tri-snRNPs and dissociates with the complex during the spliceosome activation (Figure 1; middle part)[4–6]. It is thought to be a crucial protein component regulating spliceosome assembly yet the detailed mechanism is still unclear. Here we used yeast-two-hybrid and *in vitro* binding assays that

Snu66 binds directly to an uncharacterized AAA+ ATPase Sip2 (Snu66-interacting protein 2), which associates with two other splicing factors and genetically interacts with core component of the spliceosome *PRP8* gene, suggesting that Sip2 might play an important role in pre-mRNA splicing.
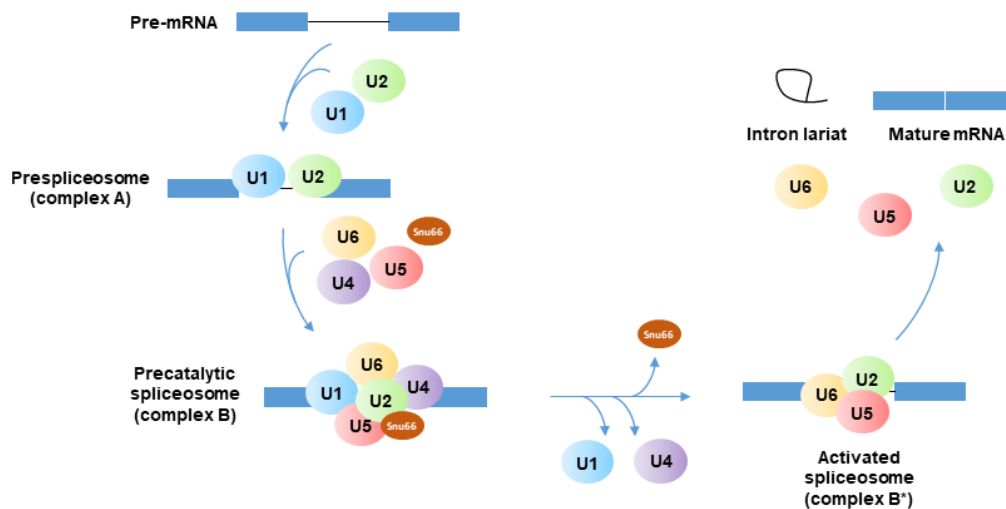

Figure 1. Schematic representation of the splicing cycle, which is a sequential assembly of components of the spliceosome involved with five snRNAs: U1, U2, U4, U5, and U6. Snu66 participates the cycle during the recruitment of preformed U4.U6/U5 tri-snRNP but dissociates with the complex prior to activation of the spliceosome with unknown mechanism.

Methodology

Strains and Genetic Techniques

Yeast strains used in this study are listed in Table 1. Yeast media, growth conditions, and genetic and molecular techniques were as described previously[7]. *SIP2* gene was deleted from the W303a wild-type and *prp8** strains using a one-step gene replacement technique[6,8]. Correct integration of deletion cassette was confirmed by PCR amplification of genomic DNA with a primer specific for *SIP2* promoter and an internal primer specific for the deletion cassette[8].

Yeast-two-hybrid Screen

For yeast two-hybrid screens, approximately 2×10^6 PJ69-7A yeast cells were transformed with a pGBDU-*SNU66*[6] and a yeast genomic two-hybrid library in pGAD construct[9]. Transformants were selected onto synthetic complete (SC)-Leu-Ura-His plates containing 40 mM 3-aminotriazole for 3–4 days. Surviving yeast colonies were picked as primary positives and restreaked on selection plates to isolate single clones. Plasmids isolated from positive clones were subjected to PCR. The resulting PCR products were purified and sequenced.

Preparation of Recombinant Proteins and GST Pulldown

GST-fused and His-tagged proteins were expressed in *E. coli* by induction with 1 mM isopropyl-D-thiogalactopyranoside (IPTG) at 37°C for 4 h. The cells were harvested and sonicated in PBST buffer (phosphate-buffered saline containing 1% Triton X-100). After centrifugation, the supernatants were collected and stored at −80 °C. For purification, the extracts were mixed and incubated with glutathione-Sepharose 4B (Amersham Pharmacia Biotech) at 4°C for 1 h. The beads were centrifuged, washed five times with excess PBST buffer, and subsequently eluted with 10 mM reduced glutathione in PBST buffer.

Results and Discussion

To gain an insight into Snu66's function, we performed a yeast-two-hybrid screen using Snu66 as bait and aimed at identification of novel interactors. We screened more than 2,500 candidate colonies in total and confirmed the interaction between Snu66 and a protein Sip2, for Snu66-Interacting Protein 2 (Figure 2A). Sip2 protein contains an evolutionarily conserved uncharacterized AAA+ ATPase domain, found in all kingdoms of life and participating in diverse cellular processes[10]. Next, we mapped the Sip2-INteracting Domain (SIND) motif in Snu66 using truncated versions of the protein and identified crucial region for the binding. Strikingly, the SIND motif is highly conserved among eukaryotic species (Figure 2B), suggesting strongly that the region is important for Snu66's function.
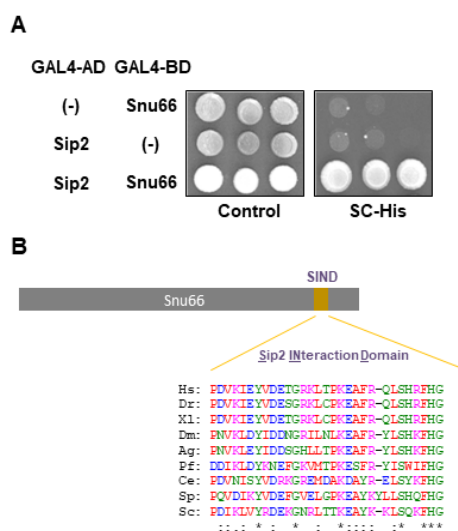

Figure 2. Identification of Sip2 as a novel interactor of Snu66 (A) Yeast-two-hybrid assay showed an *in vivo* interaction between Snu66 and Snu66-interacting protein 2 (Sip2). **(B)** Sip2-INteraction Domain (SIND) of Snu66 is highly conserved among different eukaryotic species.

The interaction between Snu66 and Sip2 observed *in vivo* by yeast-two-hybrid system could potentially occur via indirect secondary binding. To verify this hypothesis, next we constructed both Snu66 and Sip2 ORF into expression plasmids to obtain recombinant proteins for *in vitro* binding assay. Then, each construct was transformed into *E.coli BL21(DE3)* strain and expression of recombinant proteins was induced by IPTG. Subsequently, cells were collected, lysed, and His-tagged proteins of Sip2 variants purified by Ni-NTA column. To test the interaction, we performed GST-pulldown analysis using GST-Snu66-SIND as bait. Indeed, GST-fused protein copurified with full-length (FL) of Sip2 as well as two other truncated mutant variants covering AAA+ ATPase domain (Figure 3). This result strongly suggested that the interaction between Snu66 and Sip2 is direct.

The novel interaction between the splicing factor Snu66 and uncharacterized AAA+ ATPase Sip2 led us to hypothesize that the enzyme might play a role in splicing. To this end, first we performed another yeast-two hybrid screen using Sip2 as bait and identified two other splicing factors, U2 snRNP component Prp9 and the spliceosome disassembly factor Spp382, as Sip2's interactors (Figure 4A). This result reconfirmed that Sip2 might functionally play a role in splicing.
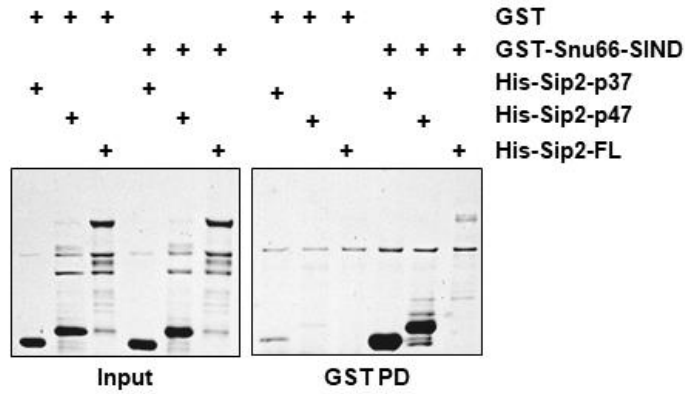
Figure 3. *In vitro* **binding assay between Snu66 and Sip2.** Coomassie-stained gel representing purified proteins used for *in vitro* GST-pulldown (PD) assays. Either GST (negative control) or GST-Snu66-SIND motif was used as bait to analyze the interaction with Sip2 variants.
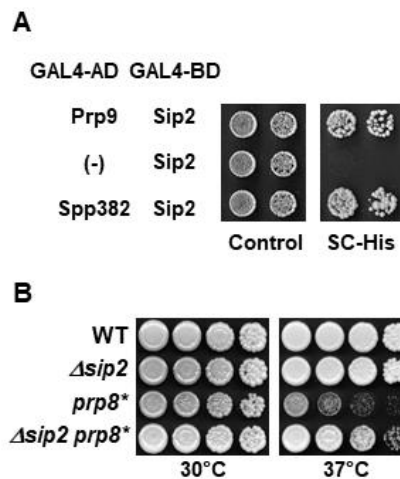

Figure 4. Sip2 potentially plays a role in splicing **(A)** Yeast-two hybrid screen identified two other splicing factors, Prp9 and Spp382, as interactors of Sip2. Ten-fold serial dilution of cells harboring indicated plasmids were spotted on plates. Cells were incubated at 30°C for 3 days. **(B)** Null mutation of *SIP2* gene suppressed temperature-sensitive phenotype of *prp8**, a mutation of core spliceosomal component *PRP8*. Ten-fold serial dilution of cells were spotted on plates containing rich media YPD. Cells were grown for 3 days at indicated temperatures. WT: wild-type.

To gain further insight into Sip2 function, we performed a synthetic interaction assay using cells lacking Sip2 and combining with a mutation of known splicing factor. Null mutation of *SIP2* gene showed no obvious phenotype at 30°C and 37°C (Figure 4B). On the other hand, mutation of an essential and core component of the spliceosome Prp8 (*prp8**) displayed a strong temperature-sensitive phenotype. Importantly, however, when we deleted *SIP2* gene from the *prp8**, the growth of cells strikingly improved (Figure 4B), indicating that both genes are functionally linked and Sip2 might play a direct role in splicing.

Table 1. Yeast strains used in this study.

Strain	Genotype	Reference
PJ69-7A	*MATa trp1-901 leu2-3, 112 ura3-53 his3-200 gal4 gal80 GAL1::HIS3 GAL2-ADE2 met2::GAL7-lacZ*	9
W303a	*MATa leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15*	K. Nasmyth
prp8*	W303a *prp8** (P1384L)	6
Δ*sip2*	W303a Δ*sip2::kanMX6*	This study
Δ*sip2 prp8**	W303a Δ*sip2::kanMX6 prp8**	This study

Conclusion

In summary, we identified an evolutionarily conserved AAA+ ATPase Sip2 as a novel interactor of tri-snRNP protein Snu66. We also mapped the Sip2's interacting area on Snu66 and found that the region is highly conserved among eukaryotes, suggesting strongly its important role. Snu66 and Sip2 interacted both *in vivo* assayed by yeast-two-hybrid analysis and directly *in vitro* by pulldown assay. Importantly, deletion of *SIP2* gene could suppress temperature-sensitive phenotype of core spliceosome component *prp8**, indicating that Sip2 might involve in pre-mRNA splicing regulation and potentially mediate the complex event of spliceosome activation.

References

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