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Protein phosphatase 2A family members (PP2A and PP6) associate with U1 snRNP and the spliceosome during pre-mRNA splicing

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ABSTRACT

Protein phosphorylation and dephosphorylation are both important for multiple steps in the splicing pathway. Members of the PP1 and PP2A subfamilies of phospho-serine/threonine phosphatases play essential but redundant roles in the second step of the splicing reaction. PP6, a member of the PP2A subfamily, is the mammalian homolog of yeast Sit4p and ppe1, which are involved in cell cycle regulation; however, the involvement of PP6 in the splicing pathway remains unclear. Here we show that PP2A family members physically associate with the spliceosome throughout the splicing reaction. PP2A holoenzyme and PP6 were found stably associated with U1 snRNP. Together our findings indicate that these phosphatases regulate splicing catalysis involving U1 snRNP and suggest an important evolutionary conserved role of PP2A family phosphatases in pre-mRNA splicing.

1. Introduction

Splicing of eukaryotic nuclear mRNA precursor transcripts (pre-mRNA) involves a two-step reaction mechanism that removes introns from pre-mRNAs transcripts. Splicing takes place in a catalytic entity termed the spliceosome [1,2]. Spliceosomes assemble on pre-mRNA substrates in a stepwise fashion involving the formation of distinct pre-splicing complexes that represent functional intermediates in the pathway [3]. During this process, a portion of the required proteins are preassembled into small nuclear ribonucleoprotein (snRNP), each centered on one or more small nuclear RNA molecule (snRNA) [4]. The spliceosomal snRNPs can be grouped into three major RNA–protein functional subunits: the U1 (12S), U2 (17S) and U4/U6, U5 tri-snRNP (25S). The U1 snRNP is the first to bind the pre-mRNA at the 5′ splice site and U2 snRNP interacts with the 3′ end of the intron prior to U4/U6, U5 assembly. In addition to the snRNP subunits, a number of non-snRNPs splicing factors play important roles during spliceosome assembly. These include a group of proteins that share a common motif consisting of repeating serine/arginine (SR) dipeptides [5].

In vitro studies have suggested that during formation of the spliceosome, several molecular rearrangements must occur which require the displacement and exchange of interacting partners within the spliceosomal complex [reviewed in [3]]. Phosphorylation of splicing factors modulates their interaction with binding partners during splicing and also appears to facilitate the spliceosome dynamics at every step of the splicing reaction [6–9]. All phosphorylation in the spliceosome detected to date takes place on serine and/or threonine residues. The phosphorylation state of splicing factors is thought to be critical for at least two events during the splicing reaction. First, spliceosome formation requires phosphorylation as SR proteins are not incorporated into the forming spliceosome *in vitro* in the presence of protein phosphatases [6,10]. Second, when *in vitro* splicing reactions are performed in the presence of phosphatase inhibitors, spliceosomes form but the catalytic steps of splicing are inhibited, suggesting that at least one dephosphorylation step is required for the progression of splicing [6]. Many snRNP-associated proteins are also phosphorylated, such as the U1-70K protein, the spliceosome-associated protein 155 (SAP155) associated with U2 snRNP, and several proteins associated with the [U4/6]/5 tri-snRNP [7,11,12]. Importantly, phosphorylation of the SR protein ASF/SF2 promotes its interaction with the U1 snRNP-associated U1-70K phosphoprotein and thereby facilitates the initial association of the U1-snRNP with the 5′ splice site [8]. Splicing also requires protein dephosphorylation; both the U1-70K and the splicing factor ASF/SF2 protein have to be dephosphorylated at a later stage, before the first step of splicing catalysis [8,9,11]. Indirect evidence from *in vitro* inhibitor experiments implicated PP1 and PP2A family member(s) in late

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steps of splicing reaction [12–16]. A later study used immunopurified recombinant phosphatases and concluded that PP1, PP2A, and to a lesser degree two PP2A family members (PP4 and PP6) play a redundant but essential role(s) in the second step of splicing [17]. However, it remains unclear if PP2A members are also involved in the first step of splicing.

We have previously shown that a phosphorylated form of PP6c specifically co-immunoprecipitates with spliceosomal snRNP proteins and that the expression and phosphorylation of spliceosomal-associated PP6c is regulated by growth stimulation in lymphocytes [18]; other studies have shown that PP6 is implicated in cell growth and signaling [19]. Here we demonstrate that PP2A family members physically associate with the spliceosome throughout the splicing reaction and that PP2A and PP6 are stably associated with U1 snRNP. We also discuss the potential role of these phosphatases in pre-mRNA splicing.

2. Material and methods

2.1. Cell culture and treatments

HeLa cells and the human embryonic kidney (HEK) 293 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM; GIBCO, BRL, Rockville, MD) supplemented with 10% fetal calf serum (FCS); (GIBCO, BRL) at 37 °C in 5% CO_2 atmosphere. Cells were grown to 75% confluence prior to cell extract preparation.

For [^{35}S]methionine labeling, HeLa and HEK 293 cells were cultured to 75% confluence and then incubated in methionine-free DMEM for 6 h in the presence of a mixture of [^{35}S]-Met/Cys (Amersham) at a concentration of 50 μCi/ml, supplemented with 5% dialyzed FCS. Cell lysates of HEK 293 or HeLa cells prepared as previously described [20]. Details of this procedure are described in the Supplemental Material.

2.2. Antibodies

The PP2A family-pan rabbit antibody U811 (PP2A-pan) was described previously and specifically reacts with the catalytic subunits of all three PP2A family members (i.e., PP2Ac, PP6c, and PP4c) [18,21]. Affinity-purified isoform-specific rabbit antibodies recognizing PP2Ac, PP4c, and PP6c were previously described [21]. Other phosphatase antibodies recognizing the PP2A structural A subunit and variable B regulatory subunit and PP1 catalytic subunits were described previously [22].

Mouse monoclonal (mAb) antibodies 3E10 [20] and Y12 react with Sm B/B′ proteins; mAb Y12 was a gift from Dr. Gideon Dreyfuss (University of Pennsylvania). Anti-U1-70K and anti-U1-A (1E1) [23,24] monoclonal antibodies were generously provided by Dr. James C. Alwine (University of Pennsylvania). mAb 9.6 reacts with the human CD2 molecule and was used as a control for the immunoprecipitation experiments [20].

2.3. Preparation of HeLa cell nuclear extracts and thymus extracts

HeLa nuclear extracts were prepared as described [25]. Human thymocytes were isolated from 20–30 g of fresh human thymus obtained from infants that had undergone open-heart surgery at the Children Hospital of Philadelphia (IRB No: 1998-3-1455). Thymus cell lysate was prepared as described in the Supplemental Material.

2.4. In vitro splicing assay

In vitro splicing assays and immunoprecipitations of splicing reactions were performed as previously described [26], except that 0.5 mg/ml tRNA was included in the washes to prevent non-specific protein interactions.

2.5. Isolation of U1 snRNP by sucrose gradient centrifugation

U1 snRNP complex of nucleoplasmic extract from Hela cells and thymocytes were isolated by sucrose gradient centrifugation as described [27]. Sucrose density gradients were prepared as described in the Supplemental Material. Thirty-four 1-mL fractions were collected using a BioComp Model 150 Gradient Fractionator (New Brunswick) at 4 °C and samples from these fractions were tested for the presence of U1 snRNP using anti-U1-A and anti-U1-70-K antibodies as described below.

2.6. Protein fractionation on MonoQ column

U1 snRNP-enriched material collected from the 12S fractions of the sucrose gradient (fractions 20–24) were pooled, diluted 3-fold with buffer A (20 mM Tris–HCl, pH 7.5, 0.1 mM EGTA, 2 mM $MgCl_2$, 1 mM DTT, 10% glycerol, 2 μg/mL pepstatin, 2 μg/mL leupetin, 2 mM benzamidine, 1 mM phenylmethylsulfonyl-fluoride, and 20 μg/mL soybean trypsin inhibitor) and applied to a Mono Q column (1 mL) equilibrated in buffer A. The column was washed (1 mL/min) with 10 mL of buffer A and then developed with a linear gradient of 0–500 mM NaCl in buffer A. One ml fractions were collected and 15 μl aliquots of each fraction were subjected to immunoblot analysis.

2.7. Immunoprecipitation and immunoblotting

Aliquots of cell lysates or 1 mL of sucrose fractions were precleared with 50 μl of a 50% slurry of protein G-Sepharose (Pharmacia) for 45 min at 4 °C with rotation followed by brief centrifugation. Immunoprecipitations and immunoblotting were performed as previously described [18]; details of these procedures are included in the Supplemental Material.

2.8. Northern blotting

Total RNA was isolated by phenol/chloroform extraction followed by ethanol precipitation. RNA was then transferred to Nytran SuPerCharge using the TurboBlotter™ System (Schleicher & Schuell Inc.) according the instructions of the manufacturer. RNA was crosslinked to the membrane by UV light (254–312 nm) for 3–5 min with a total exposure of 120 mJ. The following 5′ end-labeled oligonucleotides were used as probes: CTCCCCTGCC AGGTAAGTAT (U1), ATAAGAACAGATACTACACTTGA (U2), TACTG CCACTGCGCAAAGCT (U4), CGGAAATCTTTAGTAAAAGGCG (U5), GAACGCTTCACGAATTTGCGT (U6).

3. Results and discussion

3.1. PP2A family members physically associate with splicing products

We have previously identified a phosphorylated form of PP6c that specifically co-immunoprecipitates with spliceosomal snRNPs [18]. To further analyze the association of PP2A family members with the spliceosome and to evaluate the functional significance of these interactions, we exploited an affinity-purified PP2A family-pan antibody that specifically recognizes all three PP2A family members (PP2Ac, PP6c, and PP4) [18,21]. We tested the ability of this antibody to immunoprecipitate spliceosomal components from *in vitro* splicing reactions containing radiolabeled β-globin pre-mRNA. RNA was recovered from the immunoprecipitates and analyzed by denaturing PAGE. As shown in Fig. 1, the PP2A fam-

ily-pan antibody (PP2Ac-pan), but not a control antibody (i.e., a rabbit pre-immune serum), precipitated significant amounts of the pre-mRNA precursor, as well as the products of splicing (mRNA and lariat intron) at all time points tested during the splicing reactions (30, 60, and 120 min). The intermediates of splicing, free exon 1 and lariat-exon 2, were not detected in PP2A family-pan antibody immunoprecipitates. Likewise, relatively low amount of free exon 1 and lariat-exon 2 were found in the anti-Sm (Y12 mAb) positive control, suggesting that these intermediates are mostly removed by the adopted immunoprecipitation and washing procedure. The control serum precipitated a very small fraction of the pre-mRNA precursor (0 min time point); however, this pattern is not specific and is similar to the background noise observed in the negative controls of other experiments, which is due to the high level of radiolabeled pre-mRNA precursor used in this assay. Taken together, these results suggest that a PP2A family member(s) is associated with the spliceosome at multiple steps of splicing and that the association between the phosphatase(s) and the spliceosome is transient. The apparent transient nature of this interaction could explain why PP2A family member(s) was not detected in previous proteomic analyses of purified spliceosomes [28,29].

3.2. PP2A family members associate with U1 and U2 snRNPs

To investigate the possible association of a PP2A family member(s) with the snRNPs, immunoprecipitations were performed from HeLa nuclear extracts using the PP2A family-pan antibody. Total RNA was isolated from the immunoprecipitates and analyzed by Northern analysis using anti-sense oligonucleotide probes recognizing U1, U2, U4, U5, and U6 snRNA. U1 snRNA and to a lesser extent U2 snRNA were preferentially co-immunoprecipitated with PP2A family-pan antibody (Fig. 2A). Quantification of RNA bands using a phosphorimager revealed approximately 5-fold more U1 snRNA compared to U4 and U6 snRNAs in the PP2A family-pan antibody immunoprecipitate. No snRNAs were immunoprecipitated with the rabbit pre-immune serum, which was used as a negative control. Anti-Sm (mAb Y12), which was included as a positive control, immunoprecipitated U1, U2, U4, and U6 snRNAs. In reciprocal immunoprecipitation experiments using Hela nuclear cell extract, the anti-Sm antibodies 3E10 and Y12 co-immunoprecipitated U1-70K and PP6c (Fig. 2B). These data strongly suggest that one or more members of the PP2A family associate with U1 snRNP. The PP2A family-pan antibody co-immunoprecipitated less U2 snRNA than U1 snRNA (Fig. 2A), suggesting that either a higher proportion of U1 snRNPs contain a PP2A family member(s) or that the association between the phosphatase(s) and the U2 snRNP is not as stable. The association of PP2A family member(s) with U2 snRNP is consistent with another study that showed SAP155, a component of U2 snRNP, is an important spliceosomal substrate for PP2A/PP1 [17].

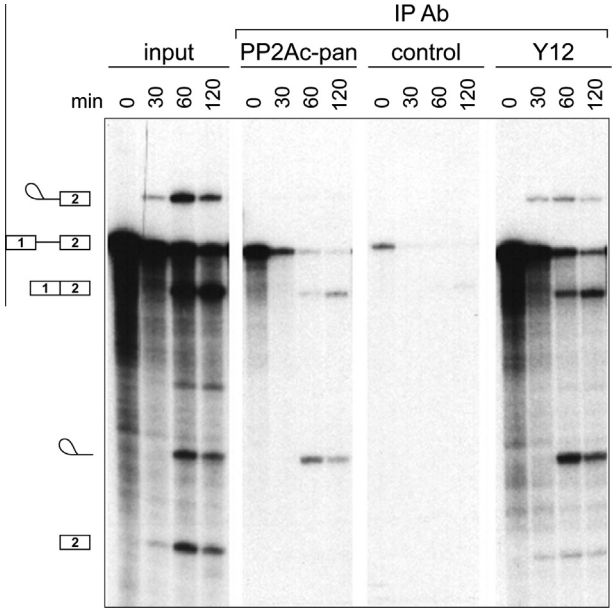

Fig. 1. PP2A family members associate with the spliceosome. *In vitro* splicing assays and immunoprecipitations of *in vitro* β-globin splicing reactions were performed using the PP2A family-pan antibody (PP2Ac-pan) at the indicated incubation times. The presence of splicesomes in the immune complexes was detected by extracting radiolabeled RNA from immunoprecipitates and separating the RNA by denaturing polyacrylamide gel electrophoresis (PAGE). The amount of input RNA shown is equivalent to 10% of the amount used for each immunoprecipitation. Anti-Sm (mAb Y12) immunoprecipitations were included as positive controls, and preimmune sera served as a negative control. An equal amount of each antibody was used in these experiments. The positions of splicing precursors, products and intermediates are indicated. The positions of the pre-mRNA, mRNA, lariat-exon 2, lariat intron, and debranched intron are shown. These results were reproducible in other independent experiments.

Further evidence for the association of a PP2A family member(s) with U1 snRNPs was obtained by immunoprecipitation experiments using U1 snRNP-rich fractions of [^{35}S]-labeled HEK 293 nuclear extracts prepared by sucrose gradient centrifugation. As shown in Fig. 2C, the U1A-specific antibody (1E1), which was used as a positive control, immunoprecipitated the entire U1 snRNP complex, as evident by the detection of U1-70K, U1-A, and Sm B/B′ proteins in the immune complex. The U1-C and other Sm proteins were excluded from the gel under these electrophoretic conditions. The pre-immune serum that was used as a negative control did not immunoprecipitate any labeled proteins. Importantly, the PP2A family-pan antibody efficiently immunoprecipitated U1-70K, U1-A, and Sm B/B′ proteins from the U1-snRNP rich nuclear cell extract (Fig. 2C). The identity of these protein bands was confirmed by immunoblotting using specific antibodies for these proteins [18]. [^{35}S]-labeled PP2Ac and PP6c proteins could not be directly identified in this immunoprecipitate because of poor labeling compared to the U1-70K and U1-A proteins but were detected in immunoblotting experiments (data not shown). These data, together with the findings shown in Fig. 2A, strongly suggest that a PP2A family member is associated directly or indirectly with U1 snRNP. In contrast, the PP1 antibody failed to immunoprecipitate U1-70K and U1-A proteins (Fig. 2C). The absence of PP1 in this immunoprecipitate suggests that PP1 is not present in the U1 snRNP particle.

3.3. U1 snRNP complexes contain PP2Ac and PP6c

To establish the identity of the PP2A family member(s) found in the U1 snRNP complexes, human thymus cell extract was fractionated by centrifugation on a 5–30% sucrose gradient and the resulting fractions were analyzed by SDS-PAGE and immunoblotting with anti-U1A, anti-U1-70K, PP2Ac-specific, and PP6-specific antibodies. As shown in Fig. 3A, U1A and U1-70K proteins are mostly concentrated in fractions corresponding to the 12S region (fractions 18–22) where U1 snRNP particles sediment as previously reported [27]. In contrast, PP2Ac was broadly distributed as revealed by the PP2A family-pan (PP2Ac-pan) and PP2Ac-specific antibodies (Fig. 3B). The distribution of PP6c as revealed using the PP6c-specific antibody appeared to be mainly concentrated in fractions 15 through 21 (Fig. 3B). However, the PP6c band revealed by the PP2A family-pan antibody had a broader distribution; this discrepancy might reflect a difference in the binding affinity of these two antibodies. In addition to PP2Ac and PP6c, a protein band revealed by the PP2A family-pan antibodies and migrating slightly faster than PP2Ac but slower than PP6c may represent PP4 (Fig. 3B).

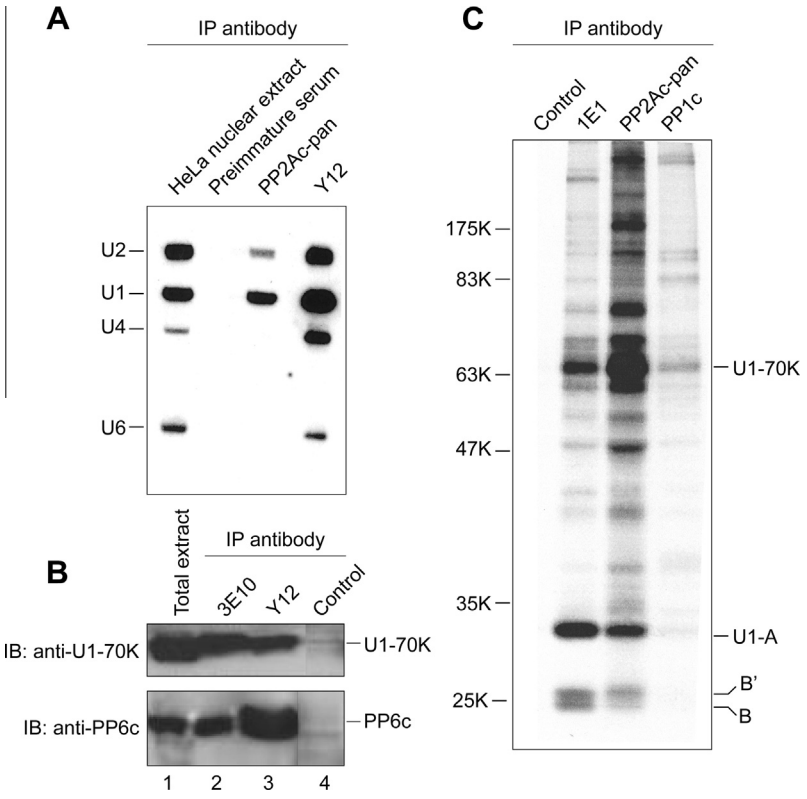

Fig. 2. PP2A family members associate with the U1 and U2 snRNPs. (A) Immunoprecipitations (IPs) of HeLa nuclear extracts were performed using either the PP2A family-pan antibody (PP2Ac-pan), the anti-Sm antibody Y12, or pre-immune serum. RNA in the immune complexes was extracted and analyzed by Northern blot analysis using antisense oligonucleotide probes. RNA was extracted from HeLa nuclear extract and loaded on the gel as a control sample. The amount corresponds to 10% of the HeLa nuclear extract used for each immunoprecipitation. The migration of various snRNAs are marked. The U5 probe was poorly labeled, and the U5 snRNA was not detected in this experiment, but was detected in other experiments. (B) Samples from HeLa cell nuclear extract were immunoprecipitated (IP) with the indicated antibodies and immunoprecipitates were analyzed by immunoblotting (IB). Lane 1: total cell extract; lane 2: IP with anti-Sm antibody (mAb 3E10); lane 3: IP with anti-Sm antibody (mAb Y12); Lane 4: IP with a control antibody. (C) [^{35}S]methionine-labeled nuclear extract from HEK 293 cells was fractionated over a 5–30% sucrose density gradient. The U1-snRNP rich fraction was immunoprecipitated (IP) with either PP2A family-pan antibody (PP2Ac-pan), PP1c antibody, pre-immune serum, or anti-U1A mAb (1E1) antibody; the latter was used to identify the protein bands of the Sm B/B′ proteins and U1 snRNP specific protein bands (U1-70K and U1-A).

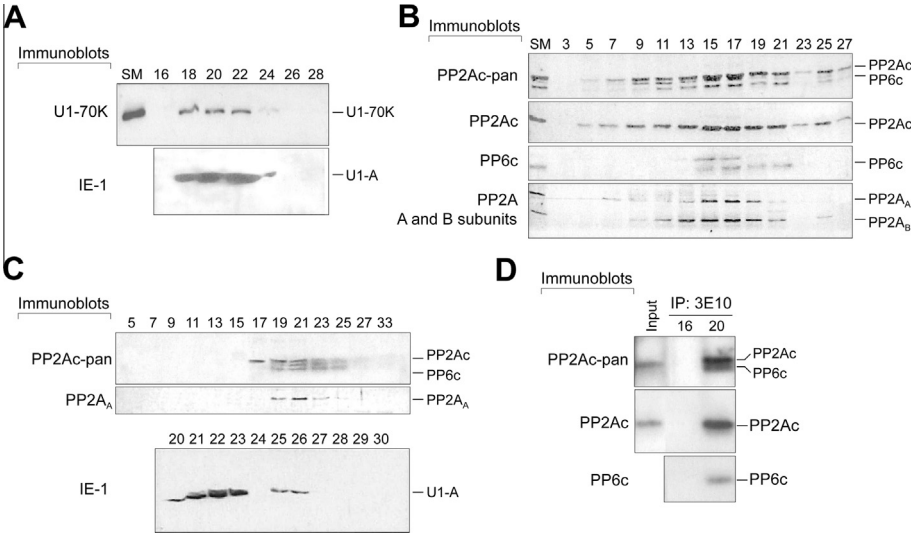

Fig. 3. Co-purification of PP6 and PP2A with U1 snRNP proteins. Human thymus extract was fractionated on a sucrose gradient (5–30%). Alternating fractions from the U1 snRNP-rich fractions (50 μg of proteins) were subjected to Western analysis using the indicated primary antibody. (A) Immunoblotting with anti-U1-70K and mAb 1E1 (U1-A). SM, designates starting material. (B) Immunoblotting with PP2A family-pan antibody (PP2Ac-pan), anti-PP2Ac and anti-PP6 specific antibodies, or antibodies specific for PP2A regulatory subunits PP2A$_A$ and PP2A$_B$. SM, designates starting material. (C) U1-snRNP-rich fractions were further purified by ion exchange Mono Q column chromatography as described in Material and Methods. Aliquots of the column fractions were analyzed by Western using PP2A family-pan antibody (PP2Ac-pan), anti-PP2A$_A$, and anti-U1A (1E1). For fraction number 24, an insufficient amount of sample was loaded on the gel thus explaining the lack of reactivity with the anti-U1A (1E1) antibody. (D) Co-immunoprecipitation of PP2Ac and PP6c with U1 snRNP. A sample of U1 snRNP-rich fractions 16 and 20 was immunoprecipitated (IP) with anti-Sm B/B′ antibody (mAb 3E10). The immunoprecipitated proteins were analyzed by Western using the PP2A family-pan antibody (PP2Ac-pan), PP2Ac- and PP6- specific antibodies.

Immunoblotting with antibodies specific for PP2A A ($PP2A_A$) and regulatory B ($PP2A_B$) subunits showed that these proteins are mostly concentrated in fractions 11 through 21, and paralleled the distribution of PP2Ac (Fig. 3B); thus suggesting that a PP2A holoenzyme (including A, B, and C subunits) is associated with the U1 snRNP complexes. Further support of the existence of a complex containing U1 snRNP, PP2Ac, and PP6c was obtained from anion exchange chromatography. Fractions collected from the 12S region of the sucrose gradient were purified by Mono Q column chromatography. Alternating fractions were probed for the presence of PP2Ac and PP6c by immunoblotting. As shown in Fig. 3C, the peaks of PP2A and U1-A overlap, but do not strictly co-elute; PP2Ac and PP6C eluted primarily in fractions 19–23. Likewise, $PP2A_A$ is eluted mostly in fractions 19–21. However, U1A is eluted mostly in fractions 21–23. PP2A phosphatases are known to associate with a plethora of regulatory subunits and/or interacting proteins, thus explaining their wide elution patterns. Complex formation of PP2Ac and PP6c with the U1 snRNP, was evaluated by immunoprecipitation and immunoblotting experiments. U1 snRNP-enriched fractions were immunoprecipitated with the anti-Sm B/B′ antibody (mAb 3E10); the immune complexes were then separated by SDS-PAGE and immunoblotted with antibodies to PP2Ac and PP6c. As shown in Fig. 3D, PP2Ac and PP6c were detected in the 3E10 immunoprecipitate using the PP2A family-pan antibody as well as antibodies specific to PP2Ac and PP6c. In contrast, no PP6c or PP2Ac was detected in the 3E10 immunoprecipitate from fraction 16, which lacks U1 snRNP proteins. These findings are consistent with the immunoprecipitation experiments using HeLa nuclear cell extract showing that the anti-Sm antibodies 3E10 and Y12 co-immunoprecipitate U1-70K and PP6c (Fig. 2B). We detected very little PP4 in the starting material. Therefore, we were unable to determine if PP4 is present in these immunecomplexes.

In other experiments, pre-treatment of cell lysates with RNase, which is known to disrupt the snRNPs core, inhibited PP6 that co-immunoprecipitated with snRNPs indicating that the association of a PP6c with spliceosomal proteins requires the presence of an RNA backbone (data not shown). However, it is unclear which type of RNA is involved in tethering the phosphatases to U1 snRNP, and we cannot rule out an involvement of endogenous pre-mRNA in these interactions in addition to the snRNA.

Taken together, our data demonstrate that PP2A family members associate with a functional spliceosome during pre-mRNA splicing and that PP2A and PP6 are associated directly or indirectly with the U1 snRNP particle. However, it remains unknown how these phosphatases are recruited to the spliceosome. The U1 snRNP could provide docking sites for PP2A and PP6 in the spliceosome and/or some of the snRNP proteins themselves may be essential spliceosomal substrates for the phosphatases. There are multiple steps at which PP6 and/or PP2A might regulate splicing catalysis associated with the U1 snRNP subunit. The action of these phosphatases may facilitate the exchange of snRNPs, whereby U1 snRNP leaves the 5′ splice site allowing recruitment of the U4/U6/U5 snRNP complex to the 5′ splice site, and hence help this spliceosome transition which is required for catalysis [8,9]. It is also possible that protein dephosphorylation by PP2A and/or PP6 participates in regulating other protein–protein interactions that must occur during splicing. Furthermore, PP2A and/or PP6 could regulate splicing-independent functions of U1 snRNP [30,31]. Further experiments will be necessary to define the precise roles of PP2A and PP6c in regulating U1 snRNP functions during pre-mRNA splicing events.

To our knowledge, this is the first report providing evidence that PP6 and a PP2A holoenzyme with its A, B, and C subunits associate with U1 snRNP, suggesting that PP2A and PP6 are important splicing factors. Our results are consistent with the several studies implicating PP2A-like phosphatases and protein phosphorylation–dephosphorylation cycles in pre-mRNA splicing and support an important evolutionary conserved role for PP2A family members in pre-mRNA splicing.

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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.bbrc.2013.09.068.

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