Prp5 bridges U1 and U2 snRNPs and enables stable U2 snRNP association with intron RNA

Yong-Zhen Xu¹, Catherine M Newnham¹,
Sei Kameoka², Tao Huang¹, Maria M
Konarska² and Charles C Query^{1,*}

¹Department of Cell Biology, Albert Einstein College of Medicine, Bronx, NY, USA and ²The Rockefeller University, New York, NY, USA

Communication between U1 and U2 snRNPs is critical during pre-spliceosome assembly; yet, direct connections have not been observed. To investigate this assembly step, we focused on Prp5, an RNA-dependent ATPase of the DExD/H family. We identified homologs of *Saccharomyces cerevisiae* Prp5 in humans (hPrp5) and *Schizosaccharomyces pombe* (SpPrp5), and investigated their interactions and function. Depletion and reconstitution of SpPrp5 from extracts demonstrate that ATP binding and hydrolysis by Prp5 are required for pre-spliceosome complex A formation. hPrp5 and SpPrp5 are each physically associated with both U1 and U2 snRNPs; Prp5 contains distinct U1- and U2-interacting domains that are required for pre-spliceosome assembly; and, we observe a Prp5-associated U1/U2 complex in *S. pombe*. Together, these data are consistent with Prp5 being a bridge between U1 and U2 snRNPs at the time of pre-spliceosome formation.

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Introduction

Removal of introns is catalyzed by the spliceosome, an ~60S assemblage of proteins and RNA. Both initial engagement of pre-mRNA by spliceosomal components and rearrangements of snRNP (small nuclear ribonucleoprotein) structures required to form an active configuration require multiple proteins of the DExD/H family that couple ATP binding/hydrolysis with structural alterations (reviewed in Schwer, 2001; Tanner and Linder, 2001). Despite much progress in understanding the role of these proteins in the progression of the splicing cycle, the exact events that they promote are still poorly understood.

One of the key necessities for spliceosome formation is juxtaposition of the 5' splice site (SS) and branch site, found at opposite ends of the intron. In both metazoan and yeast *in vitro* systems, spliceosomal components can be observed

to engage the pre-mRNA through an orderly series of interactions and rearrangements. The earliest or ATP-independent *in vitro* complexes are suggested to contain a bridging interaction from U1 snRNP at the 5'SS to SF1/BBP at the branch site (Wu and Maniatis, 1993; Abovich and Rosbash, 1997; reviewed in Reed, 2000). These interactions presumably facilitate bringing these sites to within 20 Å even in this early complex (Kent and MacMillan, 2002). However, in the first ATP-dependent transition of spliceosome assembly, the branch site–SF1/BBP interaction is disrupted and replaced by branch site–U2 snRNP interactions (reviewed in Schwer, 2001; Tanner and Linder, 2001). It is not presently known how the connection between 5'SS and branch site is maintained in this later stage.

In both mammalian and *Schizosaccharomyces pombe* systems, U2 snRNP is loosely associated in the ATP-independent E complex, but not stably engaged (Das *et al*, 2000; Huang *et al*, 2002). U2 snRNP is a 17S complex, consisting of U2 snRNA, tightly bound proteins A', B'', and the Sm heptamer, less tightly bound heteromeric protein complexes SF3a and SF3b, and probably other loosely associated factors (Krämer *et al*, 1999; Will *et al*, 2001 and references therein; reviewed in Will and Lührmann, 2001). The ATP-dependent transition to a complex in which U2 snRNP is stably bound to the intron branch site, in part through U2 snRNA·branch region base pairing, forms complex A or pre-spliceosomes (reviewed in Moore *et al*, 1993). This results in bulging of the branch site adenosine that later acts as the nucleophile in the first chemical step (Query *et al*, 1994, and references therein). Stable binding of U2 snRNP to the branch site is thought to require the DEAD-box protein Prp5, as temperature sensitive *Saccharomyces cerevisiae* Prp5 mutants fail to form pre-spliceosomes at the nonpermissive temperature (Ruby *et al*, 1993). Prp5 interacts genetically with numerous U2 components: Prp9, -11, and -21 (homologs of SF3a), Cus1p (homolog of SF3b145), Cus2p, and U2 snRNA stem-loop IIa (Ruby *et al*, 1993; Wells and Ares, 1994; Wells *et al*, 1996; Yan and Ares, 1996; Perriman and Ares, 2000). Additionally, Prp5 and the SF3a proteins are required for an ATP-dependent alteration in U2 structure (Wiest *et al*, 1996). Collectively, these data suggest a role for Prp5 in U2 function, but whether it acts as an ATPase or helicase in pre-spliceosome formation is not known.

To delineate the roles of Prp5 and ATP in early spliceosome assembly, we identified homologs of *S. cerevisiae* Prp5 and investigated their function in both human and *S. pombe* systems. Depletion and reconstitution experiments in *S. pombe* extracts demonstrate that ATP hydrolysis by Prp5 is required for stable U2 snRNP binding to the pre-mRNA branch region. Surprisingly, Prp5 contacts both U1 and U2 snRNPs, with separable domains that bind each individual snRNP. Our data support a model in which Prp5 is part of the bridge that maintains cross-intron interaction between U1 and U2 snRNPs during the formation of pre-spliceosomes.

*Corresponding author. Department of Cell Biology, Albert Einstein College of Medicine, Bronx, NY 10461-1975, USA.
Tel.: +1 718 430 4174; Fax: +1 718 430 8574;
E-mail: query@aecom.yu.edu

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Results

Identification of homologs of *S. cerevisiae* Prp5

All DEAD family proteins are highly similar within their ATPase domains; to isolate homologs of *S. cerevisiae* Prp5, we reasoned that regions external to the ATPase domain may identify it as uniquely 'Prp5' and used these sequences for BLAST searches (Altschul *et al*, 1997). This approach identified one candidate protein in a number of organisms, including humans and fission yeast, which when compared back to the *S. cerevisiae* genome had high similarity, external to the ATPase domain, only with *S. cerevisiae* Prp5 (Figure 1A and S1).

All Prp5 homologs contain the conserved ATPase/helicase domain, including the nine described signature motifs (reviewed in Caruthers and McKay, 2002; Tanner *et al*, 2003). In addition, ~100 amino-acid segments located N- and C-terminal to the ATPase domain exhibit a comparable, or higher, similarity to other DEAD family members. All homologs except *S. cerevisiae* contain RS or RD/RE dipeptide repeats near the N-terminus (e.g., 25 RS/SR and 11 RD/RE dipeptides in *H. sapiens*; seven RS/SR and six RD/RE dipeptides in *S. pombe*), a signature motif of many metazoan proteins involved in pre-mRNA splicing. Finally, all Prp5 homologs contain a conserved peptide (ExDPLDA^V/F^M) not present in other proteins represented in currently available databases.

Based on these sequence features and on functional evidence presented below relating the *S. pombe* homolog to the binding of U2 snRNP to pre-mRNA, we conclude that these are the orthologs of *S. cerevisiae* Prp5.

SpPrp5 is required for the formation of *S. pombe* complex A

To deplete and reconstitute Prp5 efficiently for examination of its role in spliceosome assembly, we prepared an *S. pombe* strain in which a TAP tag (Puig *et al*, 2001) was inserted at the 3' end of the endogenous SpPrp5 gene by homologous recombination (Figure 1B). We recently described *in vitro* analysis of complex A formation in *S. pombe* (Huang *et al*, 2002); using this system, we tested the role of SpPrp5 in U2 binding. The presence of the TAP tag did not interfere with function, insofar as complex A formed as efficiently in SpPrp5-TAP extracts as in wild-type extracts, detected by native gel electrophoresis (Figure 1C, lane 2 and data not shown). The TAP tag was then used to deplete extracts; in 1 M KCl the extract was efficiently depleted of SpPrp5, but not of snRNAs or other proteins tested, e.g. U2 SF3b155 (Figure 1B and data not shown). Extract depleted of SpPrp5 formed almost no complex A (Figure 1C, cf. lanes 4 to 2); in contrast, mock depletion of extract from untagged cells had no discernible effect (data not shown).

Figure 1 SpPrp5 is required for the formation of *S. pombe* complex A. (A) Comparison of human (upper) and fission yeast (lower) Prp5 to *S. cerevisiae* Prp5. Eight conserved motifs of DEXD/H family ATPases and the Q motif of the DEAD subfamily are indicated by black boxes (Caruthers and McKay, 2002; Tanner *et al*, 2003). Other regions of similarity are boxed in gray. *, DPLD motif unique to Prp5 homologs; RS/RD, region rich in RS, RD, and RE dipeptides. (B) (Upper) Schematic of SpPrp5-TAP fusion protein. CBP, calmodulin-binding peptide; TEV, TEV protease cleavage site; prot. A, two copies of the protein A IgG binding domain. (Lower) Analysis of SpPrp5 depletion. Untagged extracts (WT; lane 1), mock-depleted WT (lane 2), SpPrp5-TAP tagged (lane 3), and SpPrp5-TAP depleted (lane 4) were separated on 10% SDS-PAGE, Western blotted, and probed for TAP and for U2-SF3b155 as a control. Parallel Northern analysis indicated that no snRNAs were codepleted under these conditions (not shown). (C) GST-SpPrp5 reconstitutes SpPrp5-TAP depleted extracts. Intact and depleted SpPrp5-TAP extracts were incubated with ³²P-labeled pre-mRNA –/+ ATP at 30°C as indicated. GST alone, GST-SpPrp5, or Prp43p were added to depleted extracts. Samples were separated on a native 4% polyacrylamide gel, and visualized by phosphorimaging. A, U2 snRNP complexes containing pre-mRNA; H, nonspecific complexes. (D) SpPrp5-TAP is present in complexes with pre-mRNA. Extracts prepared from strains containing SpPrp5-TAP, U2-A'-TAP, or no tagged protein were incubated with ³²P-labeled pre-mRNA –/+ ATP as indicated, and complexes were affinity selected using IgG-sepharose to bind the TAP moiety. Copurifying pre-mRNA (upper); IgG-selected TAP-tagged proteins detected by Western analysis for the TAP tag (lower).

GST-SpPrp5, expressed and purified from *Escherichia coli*, restored complex A formation to the depleted extract (Figure 1C, lane 8). Consistent with activity deriving from the fusion protein, complex A complementation cofractionated with GST-SpPrp5 by gel filtration chromatography (data not shown). GST alone did not complement (lanes 5–6); likewise, the DEAH-box ATPase *S. cerevisiae* Prp43p, which is active in stimulating intron release (Martin *et al*, 2002), did not restore complex A formation, supporting the notion that complementation was specific to SpPrp5 (lanes 9–10). In addition, similarly purified mutants of GST-SpPrp5 did not complement (see below), nor did GST-SpPrp5 protein complement extracts depleted of other factors required for complex A (e.g., U2AF⁵⁹; data not shown). In all cases, complementation required incubation with ATP, as expected. We conclude that Prp5 is required for *S. pombe* complex A formation.

In similar complex A-forming reactions, SpPrp5-TAP affinity selection indicated that SpPrp5 associated with the pre-mRNA in an ATP-dependent fashion (Figure 1D, lanes 1–2). Under the same conditions, a similar amount of pre-mRNA was bound to U2 snRNP, indicated by U2-A'-TAP affinity selection (lanes 3–4). Similar levels of TAP-tagged proteins were selected in either the absence or presence of ATP. Thus, SpPrp5 becomes stably associated with the pre-mRNA, correlating with complex A formation and suggesting a direct physical role for Prp5 in the formation of this complex.

Complex A formation requires ATP hydrolysis by Prp5

GST-SpPrp5 exhibited ATP hydrolase activity, which was stimulated by poly(A) RNA and other polynucleotides (data not shown). Consistent with the previously described ATPase activity of ScPrp5 (O'Day *et al*, 1996), the maximal activity

detected was significantly less than that of Prp43 (either ScPrp43p or GST-SpPrp43; Figure 2C and data not shown).

To test whether ATP binding or hydrolysis by Prp5 is required during complex A formation, we prepared a series of alanine mutations within the ATPase domain of SpPrp5: GKT to GAT or GKA in motif I, DEAD to AEAD or DAAD in motif II, and SAT to SAA in motif III (Figure 2A). The motif I mutations disrupted ATP binding as detected by UV cross-linking (Figure 2B), and mutations in both motifs I and II disrupted ATP hydrolysis (Figure 2C), consistent with the results of such mutations in other DEXH/D proteins (reviewed in Caruthers and McKay, 2002). Mutation of DEAD → DAAD in motif II resulted in an ~20-fold enhanced crosslinking to ATP, consistent with binding but not hydrolysis (which would thereby block release) of ATP. Mutation in motif III, which in other proteins disrupts coupling of ATP hydrolysis with helicase activity, only slightly reduced ATP hydrolysis. All five mutant proteins failed to complement SpPrp5-depleted extract for complex A formation (Figure 2D, cf. lanes 7–11 to 5 or 6). Thus, ATP binding and hydrolysis by SpPrp5 are required for complex A formation. Although helicase or helicase-like activity by Prp5 has not yet been directly detected, the effect of motif III SAT mutation suggests that such activity may exist and be required for Prp5 function.

Prp5 association with pre-mRNA requires both U1 and U2 snRNPs

We next asked whether SpPrp5 association with pre-mRNA (Figure 1D) required U1 or U2 snRNPs, which we tested by RNase H degradation of U1 or U2 snRNA and compared to pre-mRNA association with U1 and U2 proteins as controls. As expected, U1-A-TAP affinity selected the pre-mRNA dependent on U1 only (Figure 3A, U1-A lane 3); U2-A'-TAP

Figure 2 ATP hydrolysis by SpPrp5 is required for the formation of *S. pombe* complex A. (A) Mutations introduced into GST-Prp5 proteins: in motif I, GKT was changed to GAT (K468A) or GKA (T469A); in motif II, DEAD was changed to AEAD (D575A) or DAAD (E576A); and in motif III, SAT was changed to SAA (T608A). (B) UV crosslinking of GST-Prp5 and mutant proteins to $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. (C) Comparison of ATPase activities of GST-SpPrp5 and mutant proteins. Values are the average from at least two parallel reactions. GST-SpPrp43 (not shown) had a similar level of ATPase activity as ScPrp43-His. (D) Ability of ATPase mutants to complement complex A formation in SpPrp5-TAP depleted extracts. Intact and depleted SpPrp5-TAP extracts were incubated with ^{32}P -labeled pre-mRNA $-/+$ ATP; either GST-SpPrp5 or mutant proteins were added to depleted extracts, as indicated. WT*, control protein whose coding sequence was altered to contain a new restriction site, but without change in amino-acid sequence. (E) ATPase mutants bind U1 and U2 snRNPs and compete effectively with WT SpPrp5 protein. GST-Prp5 proteins (WT, GAT, DAAD, SAA, or GST alone) were added to SpPrp5-TAP extract and incubated for 30 min; then snRNPs were affinity selected using the TAP tag. GKA and AEAD mutants also effectively competed for U1 and U2 binding (not shown).

Figure 3 Human and *S. pombe* Prp5 associate with U1 and U2 snRNPs. (A) SpPrp5-TAP association with pre-mRNA depends on both U1 and U2 snRNPs. Extracts prepared from strains containing SpPrp5-TAP, U1-A-TAP, or U2-A'-TAP were treated with RNase H alone (lane 2) or with RNase H and oligonucleotides targeting nt 1–14 of U1 (lane 3), nt 28–42 of U2 (lane 4), or nt 21–69 U6 snRNA as a control (lane 5). Extracts were then incubated with 32 P-labeled pre-mRNA + ATP, and complexes were affinity selected using IgG-sepharose to bind the TAP moiety. Copurifying pre-mRNA was analyzed by 10% PAGE (upper). Northern analysis of input snRNAs from SpPrp5-TAP extract after RNase H degradation (lower). The U1-A-TAP and U2-A'-TAP extracts were analyzed in parallel and showed similar levels of snRNA degradation (data not shown). (B) Affinity selection of snRNA associated with SpPrp5. Untagged (WT) or SpPrp5-TAP extracts were incubated at 30°C for 30 min and then with IgG-sepharose beads. Lanes 1 and 4, 1/4 of input. Copurifying snRNAs were analyzed by Northern blotting. (C) Affinity selection of U1 and U2 snRNP proteins by SpPrp5. Extracts from doubly tagged *S. pombe* strains containing SpPrp5-TAP and either U1-70K-3HA or U2-A'-3HA were incubated with IgG-sepharose. Copurifying proteins were analyzed by Western blotting using anti-HA antibodies. Lanes 1–2 and 7–8: 1/4 of the input extracts. Lanes 3–4 and 9–10: IgG-selected material from cells lacking any TAP-tagged protein as a control. Lanes 5–6 and 11–12: HA-tagged proteins copurifying with SpPrp5-TAP. (D) hPrp5 co-IPs U1 and U2 snRNPs. HeLa nuclear extract was incubated with –/+ ATP as indicated, and then with protein A-bound antibodies. 1/20 of input (lane 1), beads alone (lanes 2–3), anti-hPrp5 #1 against a C-terminal peptide (lanes 4–5), anti-hPrp5 #2 against the internal DPLD motif (lanes 6–7).

selected the pre-mRNA dependent on U1 and U2 (U2-A' lanes 3 and 4), consistent with the known important role of U1 in assisting U2 binding. Degrading U6 or mock RNase H treatment did not affect the association of U1-A, U2-A', or SpPrp5 with pre-mRNA, as expected (lanes 2 and 5). SpPrp5, however, had an intermediate dependency for selection of pre-mRNA—strongly dependent on U1 and partially dependent on U2 (SpPrp5p lanes 3 and 4). This suggests that SpPrp5 has a more complex mechanism of interaction with the pre-mRNA (consistent with a direct physical interaction with both U1 and U2 snRNPs demonstrated below).

Both *S. pombe* and human Prp5 associate with U1 and U2 snRNPs

To investigate physical associations with Prp5, we tested whether SpPrp5 or hPrp5 were stably associated with snRNPs in the absence of pre-mRNA. Northern analysis of SpPrp5-TAP affinity-selected material from cell extracts demonstrated that in the presence of ATP both U1 and U2 copurified (Figure 3B, lane 6). The amounts copurified represented 60–80% of total U2, and ~10% of total U1. After ATP depletion, SpPrp5-TAP only weakly copurified U2 snRNA (lane 5). Similar levels of SpPrp5-TAP were selected in either condition (data not shown; see Figure 1D). In both cases, U4, U5, and U6 were selected only at low levels, similar to the background level present in the absence of a

TAP tag (lanes 2–3). Both SpPrp5–U1 and SpPrp5–U2 associations were salt sensitive, being disrupted between 150 and 300 mM KCl, and the SpPrp5–U2 interaction was more stable than SpPrp5–U1, as monitored by native gel Western analysis (data not shown).

We confirmed interaction with both U1 and U2 snRNPs by testing whether snRNP proteins also copurified with SpPrp5. We prepared *S. pombe* strains containing SpPrp5-TAP and an HA tag on either U1-70K or U2-A' proteins, or on SpPrp43 as a control. Affinity-selected SpPrp5-TAP copurified both U1-70K and U2-A', stimulated by ATP (Figure 3C, lanes 6 and 12). In the absence of a TAP tag, no detectable amounts of these proteins were selected (lanes 3–4 and 9–10). Nor did SpPrp5-TAP copurify SpPrp43-HA from a strain expressing this pair (data not shown).

We next tested whether Prp5 associations with both U1 and U2 snRNPs were conserved in HeLa cell extracts. hPrp5 was recently detected in purified HeLa U2 snRNP (Will *et al*, 2002). Indeed, anti-hPrp5 antibody co-immunoprecipitated (co-IP'd) U2 snRNA from HeLa cell nuclear extracts; in agreement with the *S. pombe* data, however, anti-hPrp5 antibodies also co-IP'd U1 snRNA (Figure 3D). Anti-hPrp5 antibody prepared against a C-terminal peptide co-IP'd both U1 and U2 snRNAs (lanes 4 and 5), whereas antibody prepared against the conserved DPLD motif co-IP'd primarily U1 (lanes 6 and 7). This difference may be due to inaccessibility of the DPLD

motif when hPrp5 is associated with U2 snRNP, or to destabilization of U2 binding when antibody is bound to this site. Consistent with this possibility, we localized the SpPrp5-U2 interaction domain to this region (see below, Figure 5). Under the same conditions, anti-U1-A antibody co-IP'd primarily U1, and anti-U2 SF3b155 antibody co-IP'd primarily U2, as expected (data not shown). Unlike in *S. pombe* extract, hPrp5 co-IP'd U1 and U2 regardless of ATP incubation, consistent with *S. pombe* but not HeLa extracts requiring ATP-dependent dissociation of U2/5/6 prior to Prp5 binding (see Discussion) or a higher phosphatase activity in *S. pombe* extracts (unpublished). As with *S. pombe* Prp5, both hPrp5-U2 and hPrp5-U1 associations were salt sensitive, being disrupted between 150 and 300 mM KCl (data not shown).

hPrp5-U2 association was apparently significantly more stable than hPrp5-U1 association. Native gel separation of HeLa snRNPs and subsequent Western analysis indicated that hPrp5 stably associated with U2 snRNP and with complex A, in which U1 snRNP interaction is destabilized upon native gel electrophoresis. Similarly, glycerol gradient separation of HeLa nuclear extract demonstrated that a population of hPrp5 cosedimented with U2 snRNP, but not U1 snRNP (data not shown), consistent with similar observations by Will *et al* (2002). Thus, hPrp5 associates stably (through glycerol gradient and through native gel electrophoresis) with U2 snRNP and less stably (detected by IP but not by glycerol gradient) with U1 snRNP. We conclude that *S. pombe* and HeLa Prp5 associate with both U1 and U2 snRNPs.

U1 and U2 binding is independent of ATPase activity

Because we observed an enhancement of U1 and U2 binding by SpPrp5 in the presence of ATP, as well as a requirement of ATP binding and hydrolysis by SpPrp5 in complex A formation, we tested whether these were the same or different. If the same, we would expect mutants that do not bind or hydrolyze ATP to be severely defective in interaction with U1 and U2 snRNPs. Four lines of data suggest that this is not the case. It is notable here that *S. pombe* extracts initiate at a different point in the splicing cycle from other described systems, i.e., most U2 snRNP is in the form of U2/5/6 tri-snRNP and must be disassembled (in an ATP-dependent event) to become available for complex A formation and (presumably) binding to SpPrp5 (Huang *et al*, 2002), and this alone might explain the ATP requirement. (1) In a direct IP assay, in which the GST-SpPrp5 proteins were incubated with extract and then selected using the GST tag, all mutants bound U1 and U2, although less efficiently than WT protein (~40–50% of WT; data not shown). (2) In addition, when added to otherwise active extracts, all mutant proteins disrupted complex A formation (i.e., were biochemically dominant negative), again suggesting that they interact with (at least some of) SpPrp5's interaction partners (data not shown). (3) As a sensitive assay less demanding of stability, we tested whether the mutant proteins competed with endogenous WT SpPrp5 for binding U1 and/or U2 snRNPs. Increasing concentrations of GST-SpPrp5 fusion proteins were added to extract that contained SpPrp5-TAP, and then the TAP tag was used for affinity selections. GST-SpPrp5(WT) effectively competed with SpPrp5-TAP for both U1 and U2 binding, whereas GST alone had no effect (Figure 2E). GST fusion proteins harboring any of the ATPase domain mutations

described above competed for both U1 and U2 binding, similar to WT protein. Thus, the mutant proteins effectively interacted with U1 and U2. (4) Consistent with this, truncated SpPrp5 proteins lacking the ATPase domain also interacted with U1 or U2 snRNPs, although with lower efficiency compared to full-length SpPrp5 (see below, Figure 5). Therefore ATP binding/hydrolysis by SpPrp5 is not required for interaction with U1 or U2 snRNPs, although it may stabilize these associations.

SpPrp5 interacts independently with U1 or U2 snRNP

Interaction of hPrp5 and SpPrp5 with both U1 and U2 snRNPs suggested a model in which Prp5 bridges these two snRNPs. However, at least four schemes for this interaction can be envisioned (Figure 4A). In the first two, Prp5 would interact directly with only one snRNP, either U1 or U2; coassociation with the other snRNP would be due to U1-U2 interactions not directly involving Prp5. In scheme III, Prp5 would directly interact with both U1 and U2 snRNPs. Lastly, in scheme IV Prp5 might interact separately with either U1 or U2. To distinguish between these possibilities, we tested whether degradation of U1 snRNA affected SpPrp5's association with U2, and alternatively whether degradation of U2 snRNA altered SpPrp5-U1 association. RNase H-mediated degradation of the 5' half of U1 snRNA only slightly decreased the SpPrp5-U2 interaction, whereas truncated U1 did not interact with SpPrp5 (Figure 4B, lane 7). Similarly, degradation of the 5' half of U2 snRNA did not affect the SpPrp5-U1 association, whereas truncated U2 did not interact with SpPrp5 (lane 8). Degrading U6 snRNA or mock RNase H treatment had no effect on SpPrp5 associations with U1 or U2 snRNP (lanes 3–4), and similar amounts of SpPrp5-TAP were affinity selected after all RNase H treatments (lanes 3–8, Western panel). This rules out the possibility that Prp5 interaction with U1 was mediated through U2, or that its interaction with U2 was mediated through U1 (schemes I and II).

Degradation of smaller portions of U1 and U2 snRNAs, the 5' end of U1 (nt 1–14) or the central region of U2 that base pairs with the branch region (nt 28–42), yielded similar results (Figure 4B, lanes 5–6). Thus, disruptions in the small regions of U1 and U2 involved in base-pairing interactions with pre-mRNA disrupt the association with SpPrp5. We conclude that SpPrp5 can interact independently with both U1 and U2 snRNPs (schemes III and/or IV).

SpPrp5 associates with a U1/U2 di-snRNP

To test whether Prp5 interacts with U1 and U2 snRNPs simultaneously (scheme III), we separated *S. pombe* extract by glycerol gradient sedimentation and probed fractions for SpPrp5-TAP. In extract depleted of ATP, SpPrp5 sedimented in one broad peak, between 18S and 25S. In extract preincubated with ATP, two peaks of SpPrp5 were apparent, with the more abundant slow-sedimenting peak similar to sedimentation of U2 snRNP under these conditions (Figure 4C and data not shown; Huang *et al*, 2002).

Each gradient fraction was TAP affinity selected and probed for snRNAs by Northern analysis (Figure 4C). The selected snRNPs from the extract preincubated with ATP formed two peaks: both U1 and U2 were selected in similar amounts in heavier fractions, and a large amount of U2 in lighter fractions. Thus, SpPrp5 was found in two populations: one as Prp5-U2 snRNP and another as Prp5-U1/U2 di-snRNP

Figure 4 SpPrp5 interacts independently with U1 snRNP and with U2 snRNP. **(A)** Four schemes for possible interaction of Prp5 with U1 and U2 snRNPs. See text for details. Data in panel B argue against schemes I or II; and data of panels C and D and in Figure 5 support scheme III. Note that schemes III and IV are not mutually exclusive. **(B)** Northern analysis of SpPrp5-TAP affinity-purified snRNAs after RNase H-targeted degradation. Either untagged (WT) or SpPrp5-TAP extracts were treated with RNase H alone (lanes 1 and 3) or with RNase H and oligonucleotides targeting U6 snRNA as a control (lanes 2 and 4), or regions of U1 or U2 as indicated (lanes 5–8). SpPrp5-TAP-associating snRNAs were then affinity selected and analyzed by Northern blots sequentially probed for U1, U2, and U6. Truncated U1 and U2 indicate RNase H cleavage products. Aliquots of each sample were also analyzed for the amount of affinity-selected SpPrp5-TAP (Western, lower panel). **(C)** Analysis of distribution of Prp5 in *S. pombe*. Affinity selection of snRNAs associated with fractionated SpPrp5. SpPrp5-TAP extract was fractionated through a 10–30% glycerol gradient. Fractions were mixed with IgG-sepharose beads. Copurifying snRNAs were separated on a urea-denaturing gel and detected by Northern analysis (lower). Fractions were also analyzed by Western blots probed for SpPrp5-TAP (upper). Positions of 18S and 25S ribosomal RNAs from parallel gradients are indicated. **(D)** Affinity selection of a U2 snRNP protein supports the presence of U1/U2 di-snRNP. Extracts from either WT or a strain containing U2-A'-TAP were incubated with $-/+$ ATP as indicated and then with IgG-sepharose. Copurifying snRNAs were analyzed as in Figure 3B.

(Figure 4A scheme III). To confirm this result, recombinant GST-SpPrp5 was added to wild-type extract, incubated, separated by glycerol gradient, and affinity selected using glutathione beads. Northern analysis of the coselected snRNAs revealed a nearly indistinguishable profile of U1 and U2 snRNAs to that selected by the endogenous SpPrp5-TAP (data not shown).

Several lines of evidence suggest that the Prp5-associated U1/U2 di-snRNP was independent of any endogenous pre-mRNA in the extract. (1) In the presence of ATP, U2 snRNP did not detectably form complex A in the extract without addition of pre-mRNA, as indicated by native gel analysis (see Huang *et al*, 2002, Figures 2 and 3). (2) Micrococcal nuclease treatment did not alter interaction between Prp5 and U1/U2 snRNP, under conditions where $>90\%$ of an exogenous pre-mRNA was degraded (Figure S2). (3) Various Prp5 deletion mutants (Figure 5) interact only with either U1 or U2, indicating that these interactions are not dependent on pre-mRNA (otherwise, these deletion mutants would still affinity select both U1 and U2). (4) Lastly, we have

recently purified U1/U2 particles using U1 snRNA 5' end affinity selection, which is incompatible with U1 being bound to a different (or endogenous) RNA (T Huang and CC Query, unpublished).

As an independent test of U1/U2 association, we asked whether a U2 protein could affinity purify both snRNPs under these conditions. Affinity selection using U2-A'-TAP copurified the expected U2 snRNA (nearly 100%), and after ATP incubation also copurified U1 snRNA ($\sim 10\%$; Figure 4D, lanes 5–6). Similar amounts of U2-A'-TAP protein were selected regardless of ATP incubation (data not shown). Thus, a U2 protein affinity purifies a subpopulation of both snRNPs, supporting the presence of a U1–U2 association in *S. pombe*.

Different domains of SpPrp5 interact with U1 or U2 snRNP

A prediction of scheme III is that different regions of Prp5 are involved in U1 and U2 interactions. To test this, deletion mutants of SpPrp5 were prepared, expressed and purified

Figure 5 Different domains of SpPrp5 interact with U1 and U2 snRNP. (A) Schematic of GST-Prp5 deletion proteins. (B) ATPase activities of GST-SpPrp5 and deletion constructs, expressed and purified from *E. coli*. Protein A: full-length SpPrp5; B: aa171–1014; C: aa272–1014; D: aa427–1014; E: aa1–170; F: aa171–426. (C) Affinity selection of snRNA by GST-SpPrp5. Extracts were incubated with full-length or deleted SpPrp5 proteins as indicated, and then with glutathione-sepharose beads. Copurifying snRNAs were analyzed by Northern blotting. (D) Ability of deletion mutants to complement complex A formation in SpPrp5-TAP depleted extracts. Intact and depleted SpPrp5-TAP extracts were incubated with 32 P-labeled pre-mRNA as indicated. Either GST-FL-SpPrp5 (A) or deletion proteins (B–F) were added to depleted extracts.

from *E. coli* (Figure 5A), and assayed by incubation in *S. pombe* extract, subsequent affinity selection, and Northern analysis of coselected snRNAs. GST-SpPrp5 bound and coselected both U1 and U2 snRNPs, consistent with the previous affinity selection using endogenous SpPrp5-TAP protein (Figure 5C, lane 2). Deletion of the first 170aa produced a protein that bound U2, but not U1; conversely, the first 170aa alone bound U1, but not U2 (proteins B and E; Figure 5C, lanes 3 and 5). Further deletion to aa427 disrupted U2 binding, whereas aa171–426 alone did bind U2 (proteins D and F; lanes 4 and 6). We conclude that SpPrp5 contains separable regions that interact with U1 and U2.

The ability of SpPrp5 to interact with U1 and U2 was critical for its ability to complement complex A formation. Protein C (aa272–1014), which did not interact with U1 or U2, retained full ATPase activity (Figure 5B and C) but did not detectably complement depleted extract for complex A formation (Figure 5D, lane 7). Interestingly, protein B (aa171–1014) that retained ATPase activity and bound U2 but not U1, could partially restore complex A formation (30% relative to the full-length protein; Figure 5D, cf. lanes 6 to 5). This level of complex formation is similar to that observed when either 5'SS is mutated or U1 snRNA is degraded (Huang *et al*, 2002). Alternatively, the region deleted from protein B may have functions in addition to U1 binding. Other deletion proteins that were defective in ATPase activity also did not complement, as expected. We conclude that U2 interaction with Prp5 is necessary, and that the U1 interaction domain is significantly stimulatory for Prp5 function in complex A formation.

Discussion

Prp5 bridges U1 and U2 snRNPs—implications for intron and exon definition

A key consequence of spliceosome formation is juxtaposition of the 5'SS and branch site, found at opposite ends of the intron. Cross-intron bridging interactions are proposed to connect from U1 snRNP at the 5'SS to SF1/BBP at the branch site in budding yeasts or to U2AF at the pyrimidine tract in mammals (Wu and Maniatis, 1993; Abovich and Rosbash,

1997). However, in the first ATP-dependent transition of spliceosome assembly, the branch site-SF1/BBP interaction (or the pyrimidine tract-U2AF interaction) is disrupted and replaced by branch site-U2 snRNP interactions (reviewed in Staley and Guthrie, 1998; Schwer, 2001). At this stage, there has not been a model for how the connection from 5'SS to branch site is maintained. However, this could be maintained by a connection between U1 and U2 snRNPs.

Communication between U1 and U2 snRNPs has long been surmised, based on the stimulation of U2-branch site binding by the presence of a 5'SS (Robberson *et al*, 1990) and by the strong decrease in U2 binding upon U1 snRNP depletion (Barabino *et al*, 1990). Such U1–U2 communication has been predicted in spliceosome assembly models to contribute to both intron and exon definition (reviewed in Berget, 1995). Weak physical interaction between U1 and U2 was suggested in previous studies in *Xenopus* oocytes and in HeLa extracts (Mattaj *et al*, 1986; Dageron *et al*, 1992). Further hints at such an interaction came from the U12-dependent spliceosome, in which U11 and U12 snRNPs (analogs of U1 and U2) were found stably associated as a di-snRNP (Wassarman and Steitz, 1992; Frilander and Steitz, 1999). However, how U1 and U2 might communicate or interact has remained unknown.

In the evolutionarily diverse HeLa and *S. pombe* systems, we observed Prp5 association with both U1 and U2 snRNPs. These Prp5 associations are consistent with recent observations of an association of *S. cerevisiae* Prp5 with U2 snRNP (Dayeh *et al*, 2002) and purification of hPrp5 with HeLa U2 snRNP (Will *et al*, 2002), as in our studies the association with U2 is more stable than that with U1. In *S. pombe*, this association exists in a larger particle consistent with a U1/U2 di-snRNP. One possible framework in which to consider the U1–U2 association is as a subassociation of higher order penta-snRNPs, which were described recently in *S. cerevisiae* as functional splicing complexes (Stevens *et al*, 2002) and previously as physical associations in HeLa nuclear extract (Konarska and Sharp, 1988). Thus, the SpPrp5–U1/U2 di-snRNP may represent a stable subcomponent of a penta-snRNP or an intermediate in penta-snRNP assembly.

Degradation of either U1 or U2 snRNAs did not disrupt SpPrp5 association with the other, consistent with a direct and independent physical interaction with both snRNPs. Further, deletion mutants of SpPrp5 identified separable regions for direct interactions with U1 and U2 snRNPs. Both these interaction domains were necessary for optimal SpPrp5 activity in complementing complex A formation. Without the U2 interaction domain, no activity was detectable. Without the U1 interaction domain, there was a decrease (to ~30%) comparable to the decrease in complex A formation resulting from either 5'SS mutation or from U1 snRNA degradation. We conclude that Prp5 sits at an interface between U1 and U2 snRNPs and propose that it is a bridge between these two snRNPs in the formation of pre-spliceosomes (Figure 4A scheme III, and Figure 6). Consistent with this model, Prp5's ATPase activity is required at the time of pre-spliceosome formation.

Whether the U1-Prp5-U2 connection would participate in cross-exon interactions, in addition to cross-intron interactions, is unclear. In vertebrate systems, cross-exon interactions, such as from U2AF at the 3'SS, to SR proteins, to U1 snRNP at a downstream 5'SS, stimulate binding of U2 snRNP to the upstream intron. Thus, the Prp5-U1/U2 complex may also (or alternatively) represent an initial association that binds to the 3' end of introns and participate in exon definition (Robberson *et al*, 1990), consistent with the stimulatory role of U1 on U2 binding in exon definition. The strong dependence of U2 binding on the presence of U1 in HeLa extracts, even in the absence of a 5'SS (Barabino *et al*, 1990), and the early presence of U2 snRNP in mammalian and *S. pombe* complex E (Das *et al*, 2000; Huang *et al*, 2002) are both consistent with either intron or exon definition models. In either case, Prp5 would contact both U1 and U2 snRNPs and we propose that, in

either intron or exon definition, the U1-Prp5-U2 connection is important for stimulating the form of U2 snRNP active for stable binding to pre-mRNA.

Biochemical requirement for Prp5 ATPase activity

Prp5 was originally identified as a mutant in RNA biogenesis in *S. cerevisiae*. (rna5, Hutchison *et al*, 1969) and later shown to be a splicing factor (Lustig *et al*, 1986; Dalbadie-McFarland and Abelson, 1990). The presence of Prp5 homologs in *S. pombe* and a variety of metazoans suggests that Prp5 is structurally and functionally conserved. All Prp5 homologs contain the conserved 'DEAD-family' ATPase/helicase domain (reviewed in Tanner and Linder, 2001), as well as both N- and C-terminal conserved domains specific to Prp5. Excepting *S. cerevisiae* Prp5, all homologs contain an N-terminal domain rich in RS or RD/RE dipeptides, a motif found in many metazoan proteins involved in pre-mRNA splicing (reviewed in Graveley, 2000). A mammalian Prp5 was previously isolated by reactivity with antibodies prepared against nuclear components (Sukegawa and Blobel, 1995); this protein, rat HEL117, which we now identify as rat Prp5 (98.7% identical to hPrp5), was immunolocalized within speckles, colocalizing with SC35, consistent with a role as a splicing factor. More recently, hPrp5 was also immunolocalized within speckles (Will *et al*, 2002).

Genetic studies in *S. cerevisiae* placed Prp5 as a factor required early in spliceosome assembly (Ruby *et al*, 1993). Because it contains a canonical ATPase domain, Prp5's ATPase activity was expected to play a role in structural rearrangements; however, its role had not been tested. To investigate the biochemical role of Prp5, we constructed *S. pombe* strains from which we could efficiently deplete SpPrp5 and reconstitute with purified proteins, using the system recently described for analysis of *S. pombe* pre-spliceosome complex A (Huang *et al*, 2002). Such Prp5 depletion yielded extracts deficient in U2-intron binding that could be restored by add-back of purified or recombinant SpPrp5. Mutational analysis of ATPase motifs I and II demonstrated that ATP binding and hydrolysis by Prp5 is required for complex A formation. Further, mutation of motif III, SAT→SAA, suggests that helicase activity (or possibly RNase activity), although not yet directly observed, is also requisite. The *S. pombe* depletion/reconstitution system allows for future biochemical and structural analysis, which will be essential for elucidation of the consequences of Prp5 ATPase activity and of factors with which Prp5 interacts in U1 and U2 snRNPs. We conclude that Prp5 functions as an ATPase in pre-spliceosomal complex A assembly.

Role of ATP hydrolysis

In *S. cerevisiae*, Prp5 and SF3a proteins are required for an alteration of U2 structure detectable in the presence of ATP (Wiest *et al*, 1996). In the mammalian system, a structural transition in U2 is required to enable access of U2 to the branch region (Newnham and Query, 2001), resulting in deposition of SF3a and -3b proteins both 5' and 3' to the branch site (Gozani *et al*, 1996; Wang *et al*, 1998; Will *et al*, 2001).

Here, we observed several effects of ATP in relation to SpPrp5: (1) ATP was required for Prp5's association with U1 and U2 snRNPs (independent of Prp5's ATPase activity—this may be the ATP-dependent disassembly of U2/5/6 spliceosomes in *S. pombe* extracts or e.g. a phosphorylation event);

Figure 6 Schematic models of Prp5 bridging interaction with snRNPs during pre-spliceosome assembly. (Left) U1 and U2 bind substrate and are subsequently bridged by Prp5. (Right) Prp5 bridges U1 and U2 snRNPs prior to substrate binding. Hydrolysis of ATP by Prp5 is required for stable binding of U2 snRNP around the branch region. See text for details.

(2) ATP stabilized the association with U1 and U2 (dependent on Prp5's ATPase activity); (3) Prp5's ATPase activity was required for complex A formation. There are two possibilities for the natural order of these events, which relate to the mechanism of spliceosome assembly (Figure 6). The first is that when U2 binds to the intron, it is already associated with Prp5 and U1; in this case, the Prp5-U1/U2 complex represents associations that occur prior to complex A formation (Figure 6, right). The second is that U2 and U1 bind independently to the intron and then interact; in this case the Prp5-U1/U2 complex represents associations that occur after substrate binding (left). The second scenario represents established interactions that occur without ATP *in vitro*, in *S. cerevisiae* CC and mammalian and *S. pombe* E complex, which can be followed (or chased) by ATP-dependent events that include the interactions of U2 with pre-mRNA enabled by Prp5. However, *in vivo*, there is no ATP-depleted condition, and because ATP-independent conditions force progression of all interactions that can occur without ATP, this may not indicate the natural order of events. Although we cannot rule out either model, we favor the former as the predominant pathway, because Prp5 binding to U1 and U2 can occur without the addition of pre-mRNA.

Materials and methods

RNA substrate, *S. pombe* strains, media, extracts

A pre-mRNA template was derived from the gene for SF3b-p14 (SPBC29A3.07c) (sequence given in Huang *et al*, 2002). Strains of *S. pombe* used were 972 (*h*⁻), 401 (*h*⁺ *leu1-32*), and tagged derivatives of 972 described below. All strains were grown in YE medium at 30°C. Proteins of interest were tagged at their C-termini by PCR-based gene targeting as described in Huang *et al* (2002). Genomic tagging was confirmed by PCR and Western analysis. Doubly tagged strains were constructed by crossing singly tagged strains and identified by random spore analysis and Western blotting. *S. pombe* extracts and HeLa nuclear extracts were prepared as described in Huang *et al* (2002).

Expression of recombinant Prp5

SpPrp5 fragments were amplified from *S. pombe* genomic DNA by PCR, subcloned into pGEX-4T-1, and expressed in *E. coli* BL21. Alanine substitutions were created by two-stage overlapping PCR. All inserts were verified by DNA sequencing. After induction with 1 mM isopropyl- β -thiogalactopyranoside (IPTG), 11 cultures were incubated for 20 h at 17°C. Cell pellets were resuspended in 20 ml of STE500 buffer (50 mM Tris-HCl, 500 mM NaCl, 0.5 mM EDTA), incubated with lysozyme, sonicated, clarified by centrifugation, and rotated with 0.6 ml glutathione-sepharose (Amersham Pharmacia Biotech). After washing with STE500 containing 1% Triton X-100, GST-tagged proteins were eluted with 20 mM glutathione and dialyzed against buffer D (20 mM HEPES-KOH, pH 7.9, 0.2 mM EDTA, 100 mM KCl, 0.5 mM dithiothreitol (DTT), 1 mM PMSF, and 20% glycerol). Purified proteins were used at 30 nM in complementations except for 45 nM *S. cerevisiae* His₆-Prp43p (provided by A Martin and B Schwer) in Figure 1C.

Depletion of Prp5 and affinity selections

Depletions were based on TAP purification (Puig *et al*, 2001). Extracts were made from 2 l of cells; prior to dialysis against buffer D, the salt concentration was adjusted to 1 M KCl and extracts were incubated twice for 1.5 h at 4°C with IgG-sepharose beads (Pharmacia) pre-equilibrated with IPP1000 buffer (10 mM Tris-HCl

(pH 8.0), 1 M NaCl, 0.1% NP-40). Depleted extracts were then dialyzed against buffer D. The same treatment of untagged WT cells served as mock depletion.

After incubations to assemble complexes with TAP-tagged SpPrp5 or recombinant GST-tagged SpPrp5, affinity selections were performed by rotating with IgG-sepharose or glutathione-sepharose beads in 250 μ l of IPP buffer for 2 h at 4°C. Beads were washed $\times$ 5 in IPP150 for 5 min each, and then eluted by boiling in SDS loading buffer. Proteins were separated on a 10% SDS-PAGE for Western analysis. snRNAs in samples were extracted, precipitated, and separated by 10% polyacrylamide 8 M urea-denaturing gel.

Native gel and glycerol gradient analyses

Reactions to assemble *S. pombe* pre-spliceosome complex A contained 20 mM KOAc, 2 mM MgOAc₂, 0.05 mg/ml *E. coli* tRNA, 0.1 mM DTT, 0.5 U/ μ l RNasin, 1 mM ATP, 5 mM creatine phosphate, and 40% *S. pombe* extracts (Huang *et al*, 2002). Hexokinase (37 U/ml) (Roche) and 5 mM glucose were added to deplete extracts of ATP. snRNPs and complexes were analyzed by electrophoresis in native 4% acrylamide gels (80:1) run in 50 mM Tris-glycine buffer (Konarska and Sharp, 1987). *S. pombe* extracts were fractionated on 10–30% glycerol gradients in 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, and spun at 49 000 rpm for 4.25 h at 4°C in an SW50.1 rotor (Konarska and Sharp, 1987).

Crosslinking and ATPase assays

UV crosslinking was performed as described in Tanner *et al* (2003), using 1 μ g protein and 9 μ Ci [γ -³²P]ATP in 20 μ l and 254 nm light for 20 min, and then separated on 10% SDS-PAGE. ATPase assays were carried out at 30°C for 45 min and assayed both by TLC and by charcoal binding assays (O'Day *et al*, 1996; Schwer, 2000). Each reaction (20 μ l) contained 45 mM Tris-HCl (pH 8.0), 25 mM NaCl, 2.2 mM DTT, 1 mM MgCl₂, 1 mM [γ -³²P]ATP, 0.01% Triton X-100, 0.1 mM EDTA, 5 μ g of poly(A), and 225 nM protein. For charcoal assay, reactions were stopped by adding 250 μ l of 5% (w/v) charcoal suspension in 20 mM phosphoric acid. After incubation on ice for 10 min, charcoal was spun down, and the radioactivity of liberated phosphate was quantified.

Western and Northern analysis

Antiserum against hPrp5 was generated by immunizing rabbits with the peptide EELDPLDAYMEEV (aa 223–235). Antiserum against the C-terminus, RLQNSYQPTNKGRYKVL (aa 1016–1032), was a gift from J Sukegawa and was prepared against the rat Prp5 (HEL117, Sukegawa and Blobel, 1995), which is identical in this region to hPrp5. Anti-HA Western blots were probed using mAb 12AC5 (Boehringer) and sheep anti-mouse-HRP (Amersham). Western blotting against TAP was performed using rabbit anti-chicken-HRP (Pierce). Other Abs were generously provided by R Reed (SF3b155), C Lutz (U1-A), and A Krämer (SF3a60). Northern blots were probed using ³²P-labelled antisense DNA oligonucleotides.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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