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The 20kD protein of human [U4/U6.U5] tri-snRNPs is a novel cyclophilin that forms a complex with the U4/U6-specific 60kD and 90kD proteins

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ABSTRACT

Cyclophilins (Cyps) catalyze the *cis/trans* isomerization of peptidyl-prolyl bonds, a rate-limiting step in protein folding. In some cases, cyclophilins have also been shown to form stable complexes with specific proteins in vivo and may thus also act as chaperone-like molecules. We have characterized the 20kD protein of the spliceosomal 25S [U4/U6.U5] tri-snRNP complex from HeLa cells and show that it is a novel human cyclophilin (denoted SnuCyp-20). Purified [U4/U6.U5] tri-snRNPs, but not U1, U2, or U5 snRNPs, exhibit peptidyl-prolyl *cis/trans* isomerase activity in vitro, which is cyclosporin A-sensitive, suggesting that SnuCyp-20 is an active isomerase. Consistent with its specific association with tri-snRNPs in vitro, immunofluorescence microscopy studies showed that SnuCyp-20 is predominantly located in the nucleus, where it colocalizes in situ with typical snRNP-containing structures referred to as nuclear speckles. As a first step toward the identification of possible targets of SnuCyp-20, we have investigated the interaction of SnuCyp-20 with other proteins of the tri-snRNP. Fractionation of RNA-free protein complexes dissociated from isolated tri-snRNPs by treatment with high salt revealed that SnuCyp-20 is part of a biochemically stable heteromer containing additionally the U4/U6-specific 60kD and 90kD proteins. By coimmunoprecipitation experiments performed with in vitro-translated proteins, we could further demonstrate a direct interaction between SnuCyp-20 and the 60kD protein, but failed to detect a protein complex containing the 90kD protein. The formation of a stable SnuCyp-20/60kD/90kD heteromer may thus require additional factors not present in our in vitro reconstitution system. We discuss possible roles of SnuCyp-20 in the assembly of [U4/U6.U5] tri-snRNPs and/or in conformational changes occurring during the splicing process.

Keywords: chaperones; *cis/trans* isomerase; cyclophilin; peptidyl-prolyl; pre-mRNA splicing; snRNP protein

INTRODUCTION

The cyclophilins (Cyps) are a large family of proteins that bind the immuno-suppressive drug cyclosporin A (CsA), and are expressed in all organisms from bacteria to primates. Moreover, cyclophilins have been found in many subcellular compartments, including the cytosol, endoplasmic reticulum, mitochondria, chloroplasts, and the nucleus. All cyclophilins share a conserved core domain of about 110 amino acids surrounded by divergent N- and C-terminal extensions (reviewed by Walsh et al., 1992; Galat, 1993; Kunz & Hall, 1993). Cyclophilins are also enzymes with peptidyl-prolyl *cis/trans* isomerase (PPIase) activity (Fischer et al., 1989; Takahashi et al., 1989). Cyclosporin A binds with high affinity to the active site of cyclophilins and is thus an efficient competitive inhibitor of their PPIase activity (reviewed by Walsh et al., 1992). The isomerization of peptidyl-prolyl bonds

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is a rate-limiting step in protein folding, and Cyps have been shown to accelerate protein folding in vitro (Bächinger, 1987; Fischer & Schmid, 1990; Jackson & Fersht, 1991, see also Schmid et al., 1993 for review). There is increasing evidence that Cyps may also facilitate protein folding in vivo (Steinmann et al., 1991; Matouschek et al., 1995; Rassow et al., 1995). In some cases, cyclophilins have been shown to form either transient or stable complexes with specific proteins in vivo and may act as chaperone-like molecules. For example, the *Drosophila* cyclophilin NinaA forms a tight protein complex with its cellular target rhodopsin, an interaction that is important for the localization of rhodopsin to the rhabdomeres (Baker et al., 1994). In addition, CypA has been shown to bind to the gag polyprotein of HIV-1; the latter interaction is known to be required for an early step in the HIV-1 life cycle (Luban et al., 1993; Braaten et al., 1996). Furthermore, cyclophilin 40 forms a chaperone-like complex with the heat shock protein hsp90, which is required for the proper folding of steroid receptors (Duina et al., 1996).

The spliceosome, which catalyzes the removal of introns from nuclear pre-mRNA molecules, is a biochemically complex, ribonucleoprotein machinery with a highly dynamic structure. It consists of the small nuclear ribonucleoprotein (snRNP) particles, U1, U2, U4/U6 and U5, as well as an undefined number of non-snRNP splicing factors (reviewed by Guthrie, 1991; Moore et al., 1993; Krämer, 1996). The spliceosomal snRNPs contain either one (U1, U2, U5) or two (U4/U6) snRNAs and, as shown for HeLa snRNPs, more than 45 distinct proteins that are either common to all snRNP species (the Sm proteins B/B′, D1, D2, D3, E, F, and G) or specific for a given particle (reviewed in Will & Lührmann, 1997). The most biochemically complex snRNP particle is the 25S [U4/U6.U5] tri-snRNP, which is formed in the nucleus from U5 and U4/U6 snRNPs and can be isolated from nuclear extracts. In addition to the Sm proteins, tri-snRNPs from HeLa cells contain at least eight U5 snRNP-specific proteins with molecular weights of 15, 40, 100, 102, 110, 116, 200, and 220kD, the U4/U6-specific 60 and 90kD proteins, as well as five proteins characterized by molecular weights of 15.5, 20, 27, 61, and 63kD (Lauber et al., 1997 and references therein). The snRNPs, in cooperation with non-snRNP splicing factors, assemble onto a pre-mRNA intron in an ordered manner, with U1 and U2 snRNPs required for the formation of the early spliceosomal complexes E and A, respectively. Mature spliceosomes are formed by the subsequent association of the 25S [U4/U6.U5] tri-snRNP particle (reviewed by Moore et al., 1993). During spliceosome assembly, a complex RNA network consisting of several snRNA–pre-mRNA and snRNA–snRNA interactions is formed. Some of the RNAs involved in this network undergo dramatic conformational changes during the spliceosomal cycle, the most impressive example being U6 snRNA. In the 25S [U4/U6.U5] tri-snRNP, U6 snRNA is base paired together with U4 snRNA via two intermolecular helices, thereby forming a Y-shaped structure (Bringmann et al., 1984; Hashimoto & Steitz, 1984; Rinke et al., 1985; Brow & Guthrie, 1988). After association of the [U4/U6.U5] tri-snRNP with the spliceosome, the U4/U6 helices are completely dissociated prior to the first catalytic step of pre-mRNA splicing and the U6 snRNA forms new base pairing interactions with U2 snRNA (Datta & Weiner, 1991; Wu & Manley, 1991; Madhani & Guthrie, 1992; Sun & Manley, 1995), as well as the 5′ end of the intron (Sawa & Abelson, 1992; Wassarman & Steitz, 1992; Kandels-Lewis & Séraphin, 1993; Lesser & Guthrie, 1993; Sontheimer & Steitz, 1993). It is likely that the U4/U6 interaction domain reforms and that the [U4/U6.U5] tri-snRNP complex reassembles after each round of splicing (for reviews, see also Madhani & Guthrie, 1994; Nilsen, 1994).

While the dynamic nature of the spliceosomal RNA network is now well established, much less is known about changes in protein–protein interactions during the spliceosomal cycle. Although protein rearrangements may arise indirectly as a result of changes in RNA conformation, it is likewise conceivable that spliceosomal proteins could act like chaperones, directly promoting protein conformational changes and thereby contributing to the dynamics of the spliceosome. In this manuscript, we demonstrate by microsequencing and cDNA cloning that the 20kD protein of HeLa [U4/U6.U5] tri-snRNPs is a novel human cyclophilin that we denote SnuCyp-20 (Snurp Cyp-20). Consistent with its specific association with tri-snRNPs in vitro, immunofluorescence microscopy demonstrated that SnuCyp-20 is located predominantly in nuclear structures known to contain snRNPs, namely nuclear speckles. Fractionation of free proteins dissociated from [U4/U6.U5] tri-snRNPs revealed the presence of SnuCyp-20 together with the U4/U6-specific 60kD and 90kD proteins in a stable heteromeric protein complex. Coimmunoprecipitation experiments with in vitro-translated proteins further revealed a specific interaction between SnuCyp-20 and the 60kD protein. Finally, we show that isolated [U4/U6.U5] tri-snRNPs, but not U1, U2, or U5 snRNPs, exhibit peptidyl-prolyl *cis/trans* isomerase activity. Taken together, these results suggest a possible role for SnuCyp-20 in the assembly of the tri-snRNP complex and/or the spliceosome.

RESULTS

The 20kD protein of human tri-snRNPs is a cyclophilin

The 20kD protein was purified by preparatively fractionating proteins from isolated HeLa snRNPs on SDS-polyacrylamide gels as described previously (Lauber

et al., 1996). Microsequencing of the 20kD protein yielded three distinct peptide sequences. Several expressed sequence tags (ESTs) that encoded one or more of the 20kD peptide sequences were identified in the database. One EST derived from human liver (GenBank accession number T53949) was fully sequenced. The 744-bp cDNA present in this EST contains an open reading frame (ORF) encoding a putative protein of 177 amino acids with a predicted molecular weight of 19.2kD (Fig. 1). The proposed initiation codon is located at nt 14–16, and those sequences located just upstream are in agreement with the eukaryotic initiation sequence consensus (Kozak, 1987). The proposed termination codon (TAG) at nt 545–547 is followed by a polyadenylation signal (AATAAA) at nt 723–728 (Fig. 1). The identity of the 20kD cDNA was verified by three criteria. First, all of the peptide sequences obtained from the native 20kD tri-snRNP protein were found within the predicted amino acid sequence of the cDNA clone (Fig. 1). Second, the 20kD protein prepared by in vitro translation comigrated with the native 20kD protein from purified HeLa tri-snRNPs on an SDS polyacrylamide gel (Fig. 2A), demonstrating that the cDNA encodes the full-length protein. Third, antibodies raised against recombinant 20kD protein reacted specifically with a 20kD protein on immunoblots containing proteins from purified HeLa snRNPs (Fig. 2B, lane 4) or HeLa nuclear extract (Fig. 2B, lane 3).

A database search with the amino acid sequence of the 20kD tri-snRNP protein revealed that it is a novel member of the human cyclophilin family (henceforth also termed SnuCyp-20). As shown in Figure 3, SnuCyp-20 shares a strongly conserved central region with other known cyclophilins from diverse organisms. Moreover, those amino acids of CypA and CypB found to physically contact cyclosporin A (CsA) upon dimer formation (Mikol

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                                                    C CGG GTC GGA GCC   13

14  ATG GCG GTG GCA AAT TCA AGT CCT GTT AAC CCC GTG GTG TTC TTT GAT GTC AGT ATT GGC   73
1   M   A   V   A   N   S   S   P   V   N   P   V   V   F   F   D   V   S   I   G   20

74  GGT CAG GAA GTT GGC CGC ATG AAG ATC GAG CTC TTT GCA GAC GTT GTG CCT AAG ACG GCC  133
21  G   Q   E   V   G   R   M   K   I   E   L   F   A   D   V   V   P   K   T   A   40

134 GAG AAC TTT AGG CAG TTC TGC ACC GGA GAA TTC AGG AAA GAT GGG GTT CCA ATA GGA TAC  193
41  E   N   F   R   Q   F   C   T   G   E   F   R   K   D   G   V   P   I   G   Y   60

194 AAA GGA AGC ACC TTC CAC AGG GTC ATA AAG GAT TTC ATG ATT CAG GGT GGA GAT TTT GTT  253
61  K   G   S   T   F   H   R   V   I   K   D   F   M   I   Q   G   G   D   F   V   80

254 AAT GGA GAT GGT ACT GGA GTC GCC AGT ATT TAC CGG GGG CCA TTT GCA GAT GAA AAT TTT  313
81  N   G   D   G   T   G   V   A   S   I   Y   R   G   P   F   A   D   E   N   F  100

314 AAA CTT AGA CAC TCA GCT CCA GGC CTG CTT TCC ATG GCG AAC AGT GGT CCA AGT ACA AAT  373
101 K   L   R   H   S   A   P   G   L   L   S   M   A   N   S   G   P   S   T   N  120

374 GGC TGT CAG TTC TTT ATC ACC TGC TCT AAG TGC GAT TGG CTG GAT GGG AAG CAT GTG GTG  433
121 G   C   Q   F   F   I   T   C   S   K   C   D   W   L   D   G   K   H   V   V  140

434 TTT GGA AAA ATC ATC GAT GGA CTT CTA GTG ATG AGA AAG ATT GAG AAT GTT CCC ACA GGC  493
141 F   G   K   I   I   D   G   L   L   V   M   R   K   I   E   N   V   P   T   G  160

494 CCC AAC AAT AAG CCC AAG CTA CCT GTG GTG ATC TCG CAG TGT GGG GAG ATG TAG TCC AGA  553
161 P   N   N   K   P   K   L   P   V   V   I   S   Q   C   G   E   M   *          177

554 AGA CAA GAC TGA ATC AGG CCT TCC CTT CTT CTT GGT GGT GTT CTT GAG TAA GAT AAT TGG  613

614 ACT GGC CCC CGT CTT TGC TTC CCT GCC TGC TGC TGC CCC ATT TGA TCA AGA GAC CAT GGA  673

674 AGT GTC AGA GAT TCA GAA TCC AAG ATT GTC TTT AAG TTT TCC AAC TGT AAATAAAGT TTT  733

734 TTT GTA TGC GT                                                               744
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FIGURE 1. Nucleotide and predicted amino acid sequence of the human 20kD tri-snRNP protein (EST T53949). Peptide sequences obtained by microsequencing are underlined, the termination codon is marked by an asterisk, and the polyadenylation signal in the 3′ UTR is shown in bold. The full sequence is deposited in the EMBL databank (accession number AF036331).

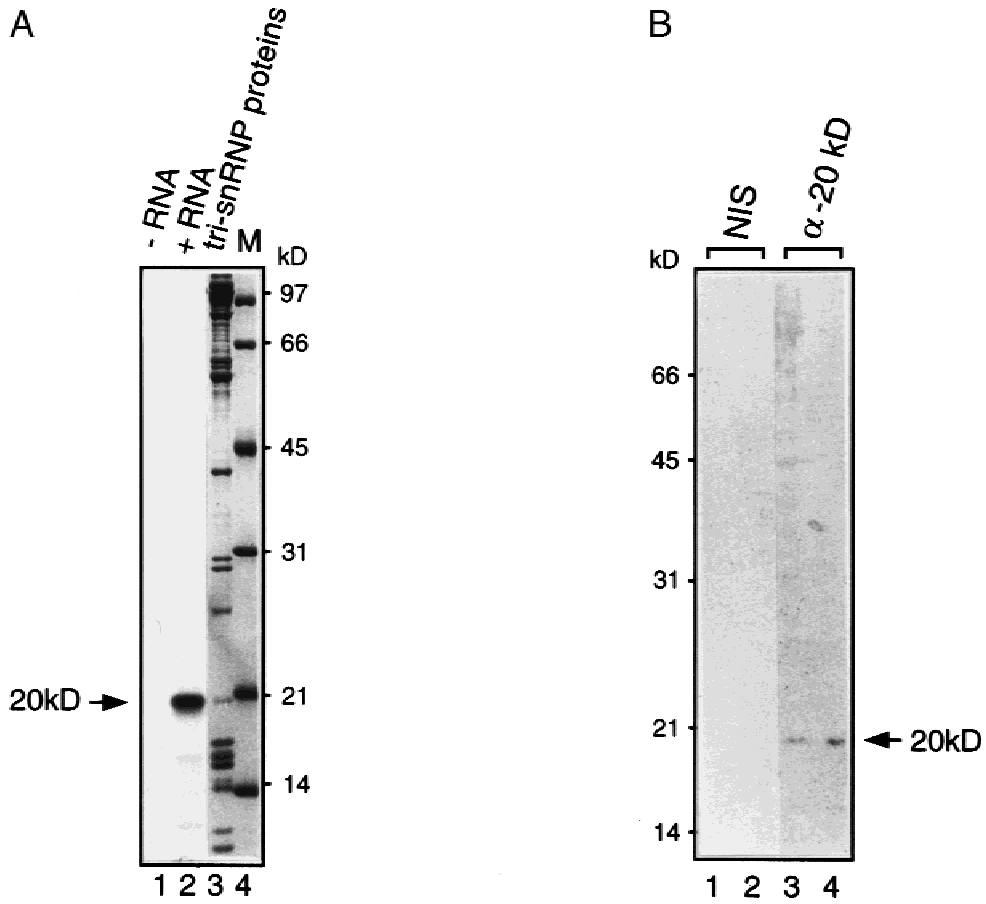


FIGURE 2. Characterization of the cDNA-encoded 20kD tri-snRNP protein (SnuCyp-20). **A:** cDNA-encoded in vitro-translated 20kD protein comigrates with native tri-snRNP 20kD protein. In vitro translation reactions containing ^{35}S methionine were performed in the absence (lane 1) or presence (lane 2) of mRNA transcribed from the 20kD cDNA. The translates were fractionated together with purified tri-snRNP proteins (lane 3) and marker proteins (lane 4) on a 13% SDS polyacrylamide gel. Proteins were visualized by fluorography (lanes 1, 2) or by Coomassie staining (lanes 3, 4) of the gel. The molecular weight of the marker proteins (in kD) is shown on the right and the position of the 20kD protein is indicated on the left. **B:** Antibodies raised against recombinant 20kD protein recognize the native tri-snRNP protein. Nuclear extract (lanes 1, 3) and purified snRNP proteins (lanes 2, 4) were separated by SDS/PAGE and electroblotted onto nitrocellulose. The membrane was immunostained with antibodies directed against the 20kD protein (lanes 3, 4) or nonimmune serum derived from the same rabbit (lanes 1, 2). The position of molecular weight markers is indicated on the left and that of the 20kD protein on the right.

et al., 1993, 1994; Thériault et al., 1993) are conserved in the SnuCyp-20 protein; only Ala_{103} (position 115 in SnuCyp-20) is changed to a serine and this residue contacts CsA only via its main chain. Comparison of the SnuCyp-20 sequence with cyclophilins from other organisms revealed an extraordinarily high degree of homology (68% identity and 81% similarity) with Cyp11 from *Caenorhabditis elegans* (Page et al., 1996). Strong homology between these two proteins is also found in regions that are not typically conserved among other cyclophilins. For example, the C-terminal 26 amino acids of human 20kD and *C. elegans* Cyp11 are 77% identical, whereas this region of SnuCyp-20 is only 23% and 38% identical to a comparable region of human CypA and CypB, respectively. This suggests that Cyp11 may be the functional counterpart of the human 20kD tri-snRNP protein in *C. elegans*.

Purified [U4/U6.U5] tri-snRNPs exhibit peptidyl-prolyl *cis/trans* isomerase activity

Identification of the tri-snRNP 20kD protein as a member of the cyclophilin family suggested that it, like other cyclophilins (see Introduction), might exhibit peptidyl-prolyl *cis/trans* isomerase activity. Because it was difficult to obtain recombinant 20kD protein in a soluble form (data not shown), we investigated whether purified tri-snRNPs possessed PPIase activity. Affinity-purified snRNPs from HeLa cells were fractionated by glycerol gradient centrifugation (see Fig. 4A for the snRNA analysis across the gradient). The distribution of SnuCyp-20 in the glycerol gradient was analyzed subsequently by immunoblotting. As shown in Figure 4B, SnuCyp-20 co-fractionated together with the 25S [U4/U6.U5] tri-snRNP complex, peaking in fractions 6–10 of the gradient (compare Fig. 4A and B). Consistent with its designation as a

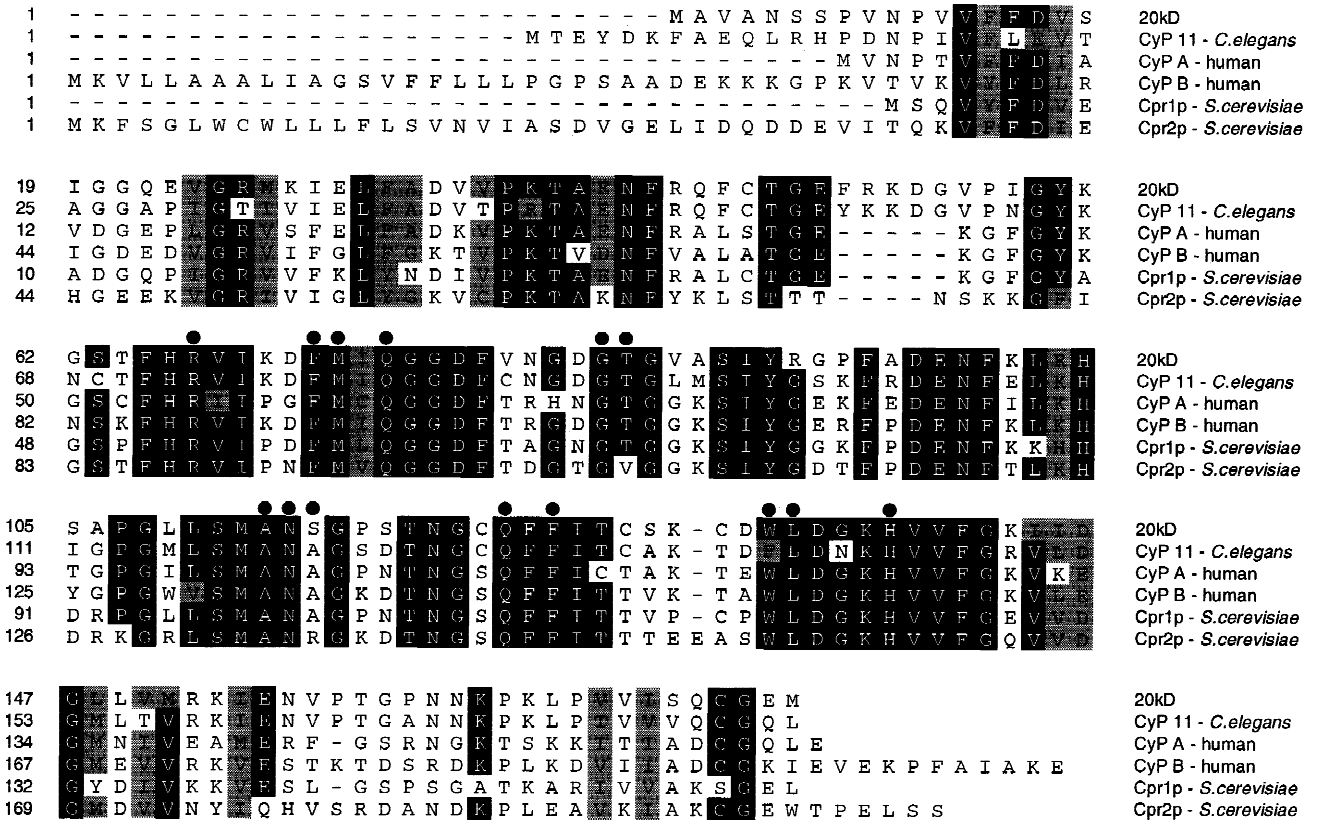


FIGURE 3. Alignment of SnuCyp-20 with selected cyclophilins. SnuCyp-20 was aligned with Cyp11 from *C. elegans* (accession number P52018), cyclophilin A and B from human (P05092 and P23284), and cyclophilin 1 and 2 from *S. cerevisiae* (P14832 and P23285) using the CLUSTAL algorithm. Amino acids that are identical in at least five of the proteins are boxed in black, whereas those that are conserved in at least five are shaded grey. Conserved amino acids are grouped as follows: (DE), (HKR), (FWY), (CILMV), and (AG). Black dots indicate amino acids that contact CsA in human CypA and CypB (Thériault et al., 1993; Mikol et al., 1994).

tri-snRNP component, essentially no SnuCyp-20 was detected in fractions 13–15 of the gradient that contain predominantly free 20S U5 snRNPs, nor was it observed in those fractions containing mainly U1 and U2 snRNPs (19–28) (Fig. 4A,B).

In parallel, we analyzed each fraction of the same gradient for PPIase activity, using the substrate Suc-Ala-Ala-Pro-Phe-4-nitroanilide (see Materials and Methods). *Cis/trans* isomerization of the alanine-proline bond of this peptide was measured spectroscopically and was expressed as the relative increase in activity over the uncatalyzed reaction [$(k_{cat}/k_o) - 1$]. As shown in Figure 4C, significant PPIase activity was detected in fractions 6–10, which coincides with the presence of the 20kD tri-snRNP protein (SnuCyp-20) as well as tri-snRNPs. Moreover, only background PPIase activity was observed at the top of the gradient or in those fractions containing 20S U5 or 12S U1 and U2 snRNPs (Fig. 4C). The PPIase activity of gradient fractions containing [U4/U6.U5] tri-snRNP could be inhibited in a concentration-dependent manner by the addition of cyclosporin A (Fig. 4D), demonstrating that the isomerase activity is due to the presence of a cyclophilin in these fractions. Because none of the other proteins present in the 25S [U4/U6.U5] tri-snRNP complex contains a domain that potentially exhibits peptidyl-prolyl *cis/trans* isomerase activity (Introduction and our unpubl. cDNA cloning data), we conclude that the PPIase activity detected in fractions 6–10 of the glycerol gradient (Fig. 4C) is by all probability due to the tri-snRNP 20kD protein (SnuCyp-20).

Subcellular localization of SnuCyp-20

Although we have thus far demonstrated that SnuCyp-20 can be coisolated with the [U4/U6.U5] tri-snRNP in a reproducible manner (Fig. 4A,B), it is not clear what fraction of total cellular SnuCyp-20 is associated with snRNPs and whether separate pools of SnuCyp-20 exist in the cell. As a first step to address this question, we prepared nuclear and cytosolic extracts from HeLa cells and determined the amount of SnuCyp-20 present in each by immunoblotting. Approximately 80% of the SnuCyp-20 was found in nuclear as opposed to cytosolic extract, demonstrating that it is predominantly localized in the nucleus. The actual amount of nuclear SnuCyp-20 may be even higher than 80% if it is considered that a certain fraction of nuclear

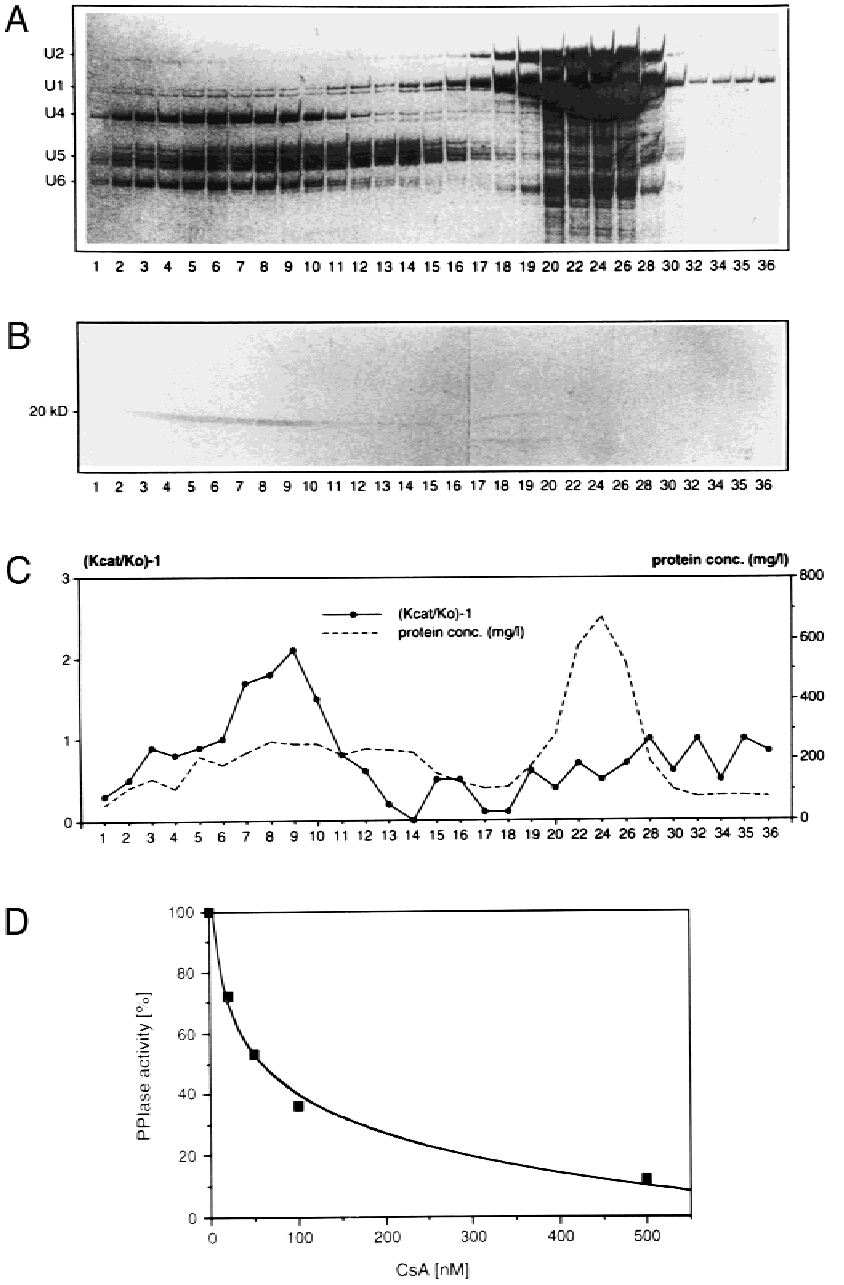


FIGURE 4. PPIase activity is associated with [U4/U6.U5] tri-snRNPs. Affinity-purified snRNP particles were fractionated on a 10–30% glycerol gradient as described in Materials and Methods. **A:** snRNAs were recovered by phenol extraction, fractionated on a 10% polyacrylamide/8 M urea gel, and visualized by silver staining. The identity of the spliceosomal snRNAs is indicated on the left. **B:** snRNP proteins fractionated on a SDS/12.5% polyacrylamide gel and subsequently transferred to nitrocellulose. The 20kD tri-snRNP protein (SnuCyp-20) was visualized by immunostaining with anti-20kD antibodies. **C:** Gradient fractions were also assayed for PPIase activity as described in Materials and Methods. The graph shows the catalytic activity (solid line) expressed as relative increase over the negative control, which was performed in the absence of protein [$(k_{cat}/k_o) - 1$] and the protein concentration (μg/mL, dashed line) as determined by a dye binding assay (Bradford, 1976). **D:** In a separate experiment, the catalytic activity of 14 pmol/100 μL of tri-snRNP was assayed in the presence of 0, 25, 50, 100, and 500 nM cyclosporin A. The graph shows the relative activities, where activity in the absence of cyclosporin A was defined as 100%.

snRNPs and thus also SnuCyp-20 leaks from the nuclei during the fractionation of nuclei and cytosol. In contrast, consistent with its reported cytoplasmic location (Galat, 1993), cyclophilin A was found almost exclusively in cytosolic extracts as investigated by immunoblotting with a monoclonal anti-CypA antibody (data not shown).

Next, we investigated the steady-state distribution of SnuCyp-20 in HeLa cells by immunofluorescence microscopy using an antiserum raised against recombinant SnuCyp-20. As shown in Figure 2B, this antiserum reacted predominantly with the 20kD tri-snRNP protein on immunoblots and exhibited only negligible crossreactivity with other nuclear proteins. In addition, we verified that this antiserum does not crossreact with recombinant cyclophilin A on immunoblots (data not shown), suggesting that the antibodies are directed predominantly against sequences of the 20kD protein not present in other cyclophilins. Although only a very faint background staining was seen with pre-immune serum (Fig. 5A), predominantly nuclear staining and a low level of cytoplasmic staining was observed with anti-SnuCyp-20 antibodies. A similar, though weaker, staining pattern was also observed with a rabbit antiserum raised against a synthetic peptide derived from the C-terminal sequence of the SnuCyp-20 protein (data not shown). The nuclear staining obtained with the anti-SnuCyp-20 antibody was not evenly distributed, but rather showed a number of

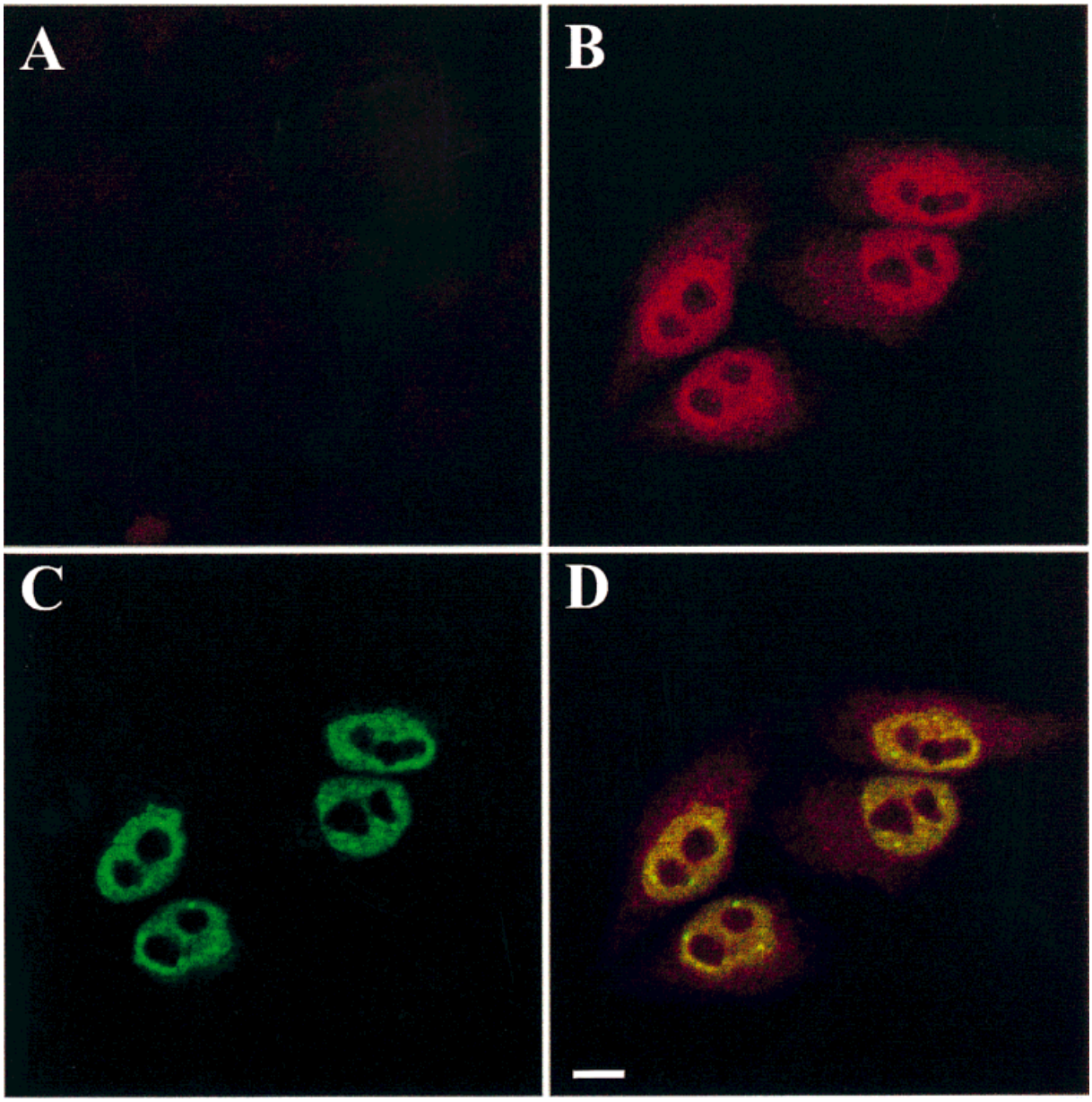


FIGURE 5. Immunofluorescence staining of SnuCyp-20 in HeLa cells. HeLa cells were stained with SnuCyp-20 preimmune serum (**A**) or double-stained with antibodies specific to SnuCyp-20 (**B**) and the monoclonal anti-Sm antibody Y12 (**C**). The red (B) and green (C) fluorescence was recorded independently and combined in an overlay image (**D**), leading to the yellow staining of structures decorated by both antibodies. The bar in D indicates 5 μm.

more intensely stained dots above the less intense general staining of the nucleoplasm and was completely absent from the nucleoli. To compare this staining pattern with the distribution of snRNPs, HeLa cells were double-stained with anti-SnuCyp-20 antibody (Fig. 5B) and the monoclonal anti-Sm antibody Y12 (Fig. 5C), which reacts predominantly with typical snRNP-containing structures referred to as nuclear speckles (Spector, 1993). The confocal overlay image shown in Figure 5D revealed a colocalization of SnuCyp-20 and snRNPs inside the nucleus, mainly in nuclear speckles (yellow spots), whereas faint but reproducible cytoplasmic staining was observed only with the anti-SnuCyp-20 antibody (red). Taken together, these data suggest an association of SnuCyp-20 with snRNP-containing structures in the nucleus and possibly a pool of non-snRNP-associated SnuCyp-20 in the cytoplasm.

SnuCyp-20, but not CypA, interacts specifically with [U4/U6.U5] tri-snRNPs

Next, we were interested in investigating the specificity of the interaction of SnuCyp-20 with the [U4/U6.U5] tri-snRNP complex. For this purpose, in vitro-translated [^{35}S] methionine-labeled SnuCyp-20 was mixed with increasing amounts of either gradient-purified 25S [U4/

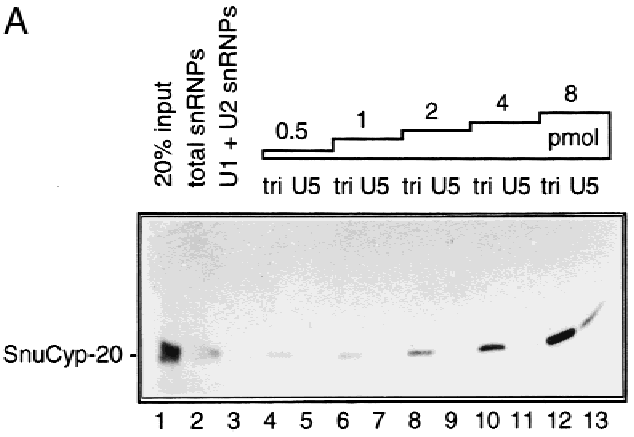
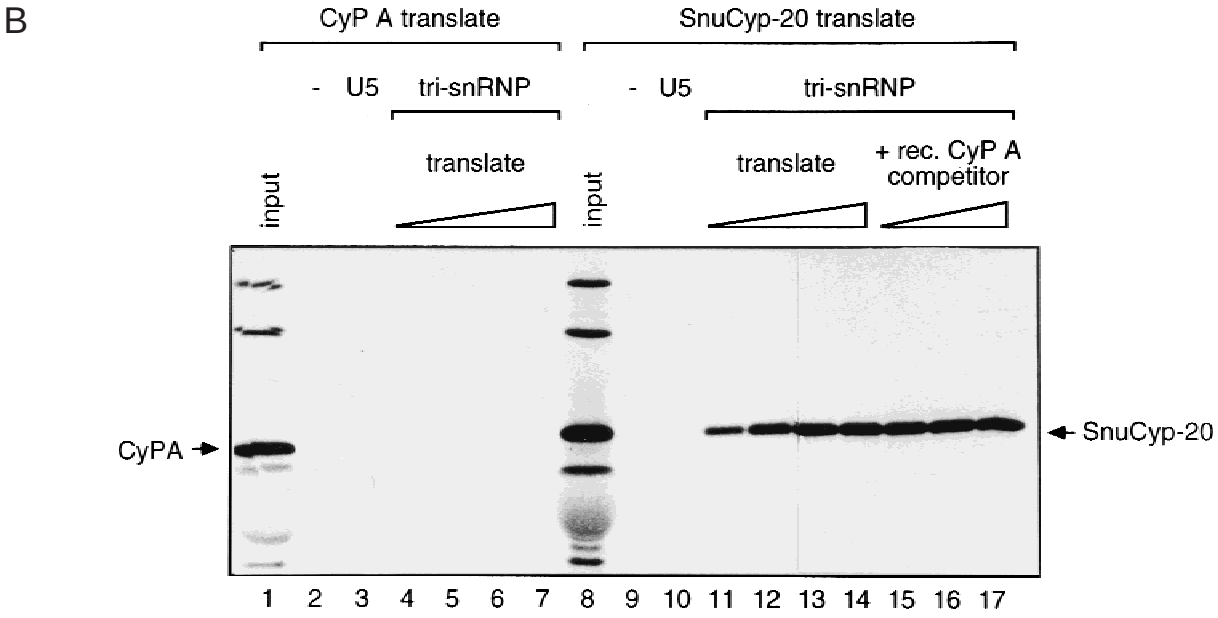


FIGURE 6. Specificity of the association of SnuCyp-20 with snRNP particles. **A:** SnuCyp-20 interacts specifically with tri-snRNP particles; 0.1 pmol of in vitro-translated, ^{35}S-labeled SnuCyp-20 was incubated with total snRNPs (lane 2), U1 and U2 snRNPs (25 pmol total, lane 3), or increasing amounts, as indicated, of glycerol gradient-purified U5 snRNP (U5, lanes 5, 7, 9, 11, 13) or [U4/U6.U5] tri-snRNP (tri, lanes 4, 6, 8, 10, 12). snRNPs were precipitated with Y12 anti-Sm antibodies, separated on a 13% SDS/polyacrylamide gel and visualized by fluorography. In lane 1, in vitro-translated protein equivalent to 20% of the amount used in the precipitations is shown. **B:** Cyclophilin A does not interact with snRNP particles. In vitro-translated Cyp A (lanes 2–7) or SnuCyp-20 (lanes 9–17) was incubated in the absence of snRNPs (lanes 2, 9, respectively), or with 0.35 pmol U5 snRNP (lanes 3, 10) or with 0.35 pmol tri-snRNPs (lanes 4–7 and 11–17). The amount of translate used was 3 fmol (lanes 4, 11), 6 fmol (lanes 5, 12), 12 fmol (lanes 6, 13), and 18 fmol (lanes 2–3, 7, 9–10, 14–17). In lanes 15–17, 0.9 pmol, 9 pmol, and 90 pmol of recombinant Cyp A (Boehringer) was added as competitor. snRNPs were immunoprecipitated with Y12 and analyzed as in A. Lanes 1 and 8 show the translation reactions equivalent to 9 fmol of the respective protein.



U6.U5] tri-snRNPs or 20S U5 snRNPs. Association with snRNPs was monitored by coprecipitation with monoclonal antibody Y12, which reacts with the core snRNP Sm proteins, but not with native SnuCyp-20. As shown in Figure 6A, significant amounts of SnuCyp-20 could be coprecipitated after incubation with tri-snRNPs, but not with U5 snRNPs (compare pairwise lanes 4–13). The low level of SnuCyp-20 precipitation observed at the highest U5 snRNP concentration (lane 13) is most likely due to the presence of contaminating tri-snRNPs in the U5 snRNP preparation. In addition, coprecipitation of in vitro-translated SnuCyp-20 was not observed with U1 or U2 snRNPs (Fig. 6A, lane 3). The ability of exogeneous SnuCyp-20 to bind [U4/U6.U5] tri-snRNPs suggests that either the endogeneous SnuCyp-20 is present substoichiometrically in isolated tri-snRNPs, or that there may be an exchange of in vitro-translated proteins with endogeneous SnuCyp-20. To determine whether tri-snRNP association is specific for SnuCyp-20, as opposed to other members of the cyclophilin family, we also investigated whether human CypA could interact with purified tri-snRNP complexes. As shown in Figure 6B, coprecipitation of in vitro-translated CypA was not observed after incubation with tri-snRNPs (lanes 4–7). Further, CypA was not able to compete for the interaction of SnuCyp-20 with tri-snRNP complexes, even when present in a 5,000-fold molar excess (Fig. 6B, lanes 11–17). Thus, despite the high overall similarity of SnuCyp-20 with other human cyclophilins (see Fig. 2), its interaction with the tri-snRNP complex appears to be strikingly specific.

SnuCyp-20 cofractionates together with the U4/U6-specific 60kD and 90kD proteins in a heteromeric complex

The association of SnuCyp-20 with [U4/U6.U5] tri-snRNPs, but not 20S U5 snRNPs, suggests that it

interacts directly with U4/U6 snRNA or, more likely, with one or more proteins that are present in the tri-snRNP complex, but not in 20S U5 snRNPs (see Introduction for a compilation of tri-snRNP proteins). In an effort to identify likely protein interaction partners of SnuCyp-20, stable heteromeric protein complexes were dissociated from [U4/U6.U5] tri-snRNP complexes in the presence of chaotropic salts. Chaotropic salts, such as sodium thiocyanate (NaSCN), are known to disrupt weak protein–protein interactions, whereas relatively stable protein complexes remain intact. Specifically, purified tri-snRNPs were incubated in the presence of 0.4 M NaSCN and then fractionated on glycerol gradients containing 0.4 M NaSCN. Figure 7A shows the protein pattern of the top 10 fractions of such a gradient. In Figure 7B, proteins from an equivalent gradient were fractionated by electrophoresis on a lower percent SDS/

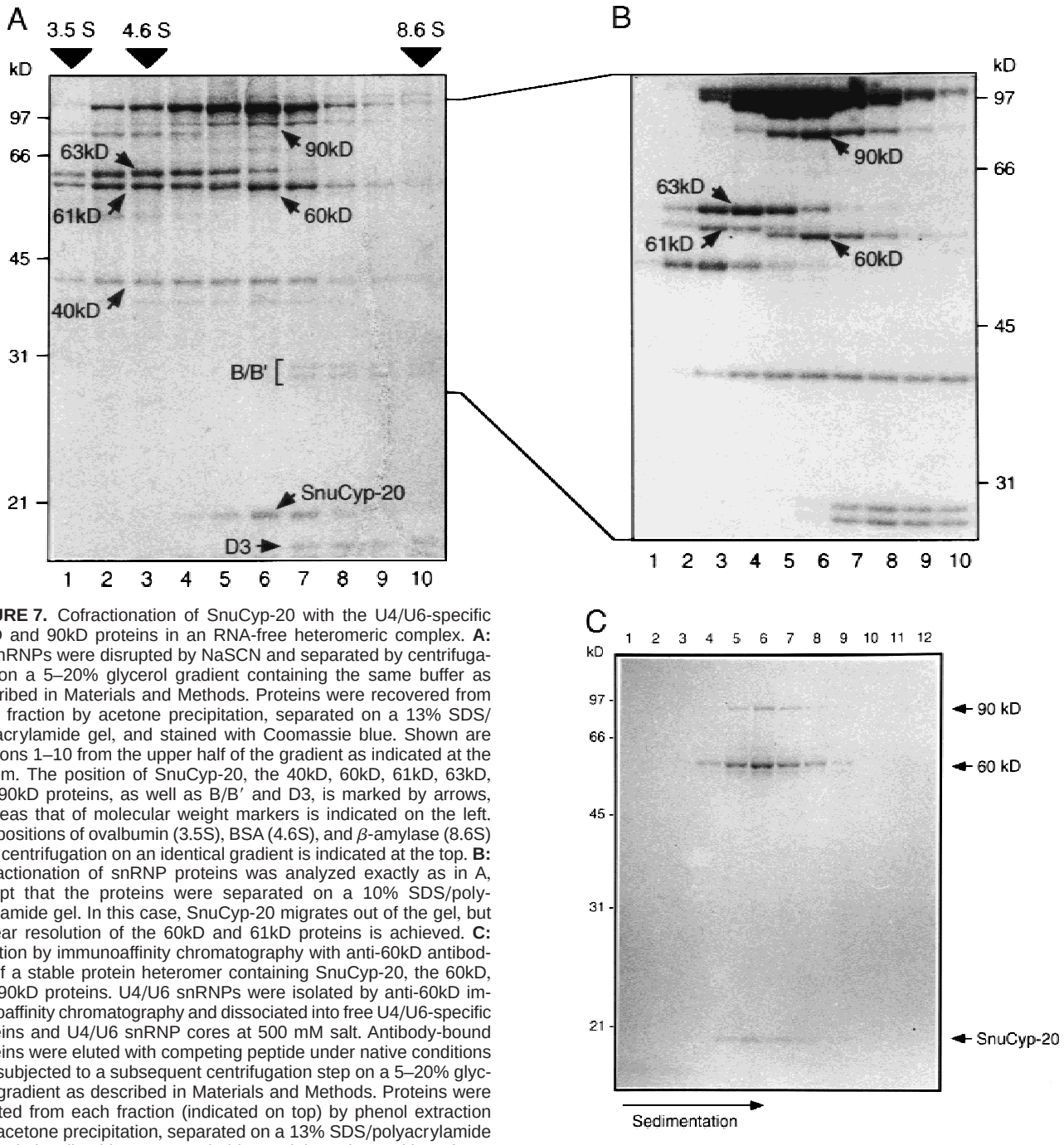


FIGURE 7. Cofractionation of SnuCyp-20 with the U4/U6-specific 60kD and 90kD proteins in an RNA-free heteromeric complex. **A:** Tri-snRNPs were disrupted by NaSCN and separated by centrifugation on a 5–20% glycerol gradient containing the same buffer as described in Materials and Methods. Proteins were recovered from each fraction by acetone precipitation, separated on a 13% SDS/polyacrylamide gel, and stained with Coomassie blue. Shown are fractions 1–10 from the upper half of the gradient as indicated at the bottom. The position of SnuCyp-20, the 40kD, 60kD, 61kD, 63kD, and 90kD proteins, as well as B/B′ and D3, is marked by arrows, whereas that of molecular weight markers is indicated on the left. The positions of ovalbumin (3.5S), BSA (4.6S), and β-amylase (8.6S) after centrifugation on an identical gradient is indicated at the top. **B:** Cofractionation of snRNP proteins was analyzed exactly as in A, except that the proteins were separated on a 10% SDS/polyacrylamide gel. In this case, SnuCyp-20 migrates out of the gel, but a clear resolution of the 60kD and 61kD proteins is achieved. **C:** Isolation by immunoaffinity chromatography with anti-60kD antibodies of a stable protein heteromer containing SnuCyp-20, the 60kD, and 90kD proteins. U4/U6 snRNPs were isolated by anti-60kD immunoaffinity chromatography and dissociated into free U4/U6-specific proteins and U4/U6 snRNP cores at 500 mM salt. Antibody-bound proteins were eluted with competing peptide under native conditions and subjected to a subsequent centrifugation step on a 5–20% glycerol gradient as described in Materials and Methods. Proteins were isolated from each fraction (indicated on top) by phenol extraction and acetone precipitation, separated on a 13% SDS/polyacrylamide gel and visualized by Coomassie blue staining. The position of molecular weight markers is indicated on the left and that of SnuCyp-20, the 60kD, and 90kD proteins on the right.

polyacrylamide gel in order to better resolve proteins in the 60kD range. The identity of the 20kD (SnuCyp-20), 60kD, and 90kD proteins in both gradients was verified by immunoblotting experiments (data not shown). As shown in Figure 7A, SnuCyp-20 peaked in fractions 5–7, corresponding to an estimated sedimentation coefficient of 6.5S. Because monomeric SnuCyp-20 is expected to exhibit a much smaller S-value (e.g., the 45kD marker protein ovalbumin with an S-value of 3.5S migrated at the top of a parallel gradient), its faster than expected sedimentation behavior indicates that it is present in a larger complex. As stated above, SnuCyp-20 most likely stably associates with tri-snRNP proteins not present in U5 snRNPs, such as the 15.5, 27, 60, 61, 63, and 90kD protein. The 15.5kD protein is found in the top fractions of the gradient (not shown), whereas the 27kD protein is consistently lost during this procedure. The 61kD and 63kD proteins peaked in fractions 2–4 (Fig. 7A,B), and thus sedimented as expected for monomers of a similar molecular weight (e.g., the 66kD marker protein BSA with an S-value of 4.6S peaks in fraction 3 of a parallel gradient). In contrast, the 60kD protein, which peaked in fractions 5–7, sedimented significantly faster than other proteins of comparable size. The 90kD protein, also found in fractions 5–7, sedimented somewhat faster than expected for a monomeric protein of this size (the molecular weight calculated from its amino acid sequence is only 77.5kD; Lauber et al., 1997). Significantly, the 20, 60, and 90kD proteins all peaked in fractions 5–7 (Fig. 7A,B), suggesting that they may form a complex with each other. Indeed, the observed sedimentation rate (approximately 6.5S) agrees reasonably well with this assumption; the added molecular weight of the three proteins (155kD) would give a theoretical sedimentation coefficient of 7.3S, assuming a globular shape of the complex (Eason, 1984). The alternative possibility that SnuCyp-20 migrates in fractions 5–7 of the gradient due to association with the 100 or 102kD proteins (see Fig. 7A) cannot be formally excluded, but is unlikely because these proteins are present in purified 20S U5 snRNPs, and binding of SnuCyp-20 to U5 snRNP particles was not observed by the coimmunoprecipitation experiments shown in Figure 6. It is important to note that, under the experimental conditions that we have employed for gradient centrifugation, the canonical Sm core proteins remain associated with the U5 and U4 snRNAs and migrate as an RNP particle in fractions 7–12 of the gradient (Fig. 7A,B, and data not shown; the Sm proteins B/B′ and D3 are marked in Fig. 7A). This suggests that the 20, 60, and 90kD proteins cofractionate, most likely as part of an snRNA-free heteromeric protein complex.

The evidence provided above, that SnuCyp-20 cofractionates as a tight complex together with the U4/U6-specific 60kD and 90kD proteins, was further corroborated by the following experiment. U4/U6 snRNPs were isolated from a mixture of anti-m_3G-purified snRNPs by immunoaffinity chromatography with an antibody raised against a synthetic peptide from the U4/U6-specific 60kD protein, essentially as described by Lauber et al. (1997). The affinity matrix-bound U4/U6 snRNPs were then washed extensively with buffer containing 0.5 M KCl. Under these conditions, the U4/U6-specific proteins dissociate from the U4/U6 snRNP cores, which contain only Sm proteins in addition to the U4 and U6 snRNAs. Although most of the U4/U6 snRNP cores are found in the high-salt washes (data not shown), the 60kD protein, together with additional stably associated proteins, is retained on the antibody column. The U4/U6-specific proteins are subsequently eluted from the antibody column by an excess of the synthetic peptide under high-salt conditions (0.5 M KCl). The rationale of this experiment is that proteins other than the 60kD proteins that are eluted by the peptide from the immunoaffinity column must be associated with the 60kD protein. The peptide eluate of the column was subjected briefly to glycerol gradient centrifugation in order to separate the proteins from the peptide and the protein composition of the various fractions was analyzed by SDS/PAGE. Significantly, only SnuCyp-20 and the 90kD protein were eluted together with the 60kD protein from the affinity column and comigrated in the glycerol gradient (Fig. 7C). No U4/U6 RNA or Sm proteins were detected in the gradient fractions (Fig. 7C and data not shown). In view of our observations that the anti-60kD antibody does not crossreact with SnuCyp-20 or the 90kD protein (see Fig. 8A), these results demonstrate that SnuCyp-20 forms an RNA-free, stable heteromeric complex with the U4/U6-specific 60kD and 90kD proteins.

SnuCyp-20 interacts directly with the U4/U6-specific 60kD protein

The availability of cDNAs encoding SnuCyp-20 and the 60kD and 90kD proteins prompted us to investigate in more detail possible interactions between these proteins in vitro. For this purpose, we mixed individual proteins, prepared by in vitro translation, in different combinations and analyzed whether SnuCyp-20 and the 90kD proteins could be coprecipitated with antibodies directed against the 60kD protein. These antibodies reacted with in vitro-translated 60kD, but not with the SnuCyp-20 or 90kD proteins in the absence of 60kD protein (Fig. 8, lanes 4–6 and 10). Interestingly, SnuCyp-20 could be coprecipitated with the 60kD protein, both in the presence and absence of the 90kD protein (Fig. 8, lanes 8 and 9), suggesting that SnuCyp-20 and the 60kD protein interact directly with one another. This interaction is stable even at higher salt concentrations (e.g., 300 mM, data not shown). When increasing concentrations of SnuCyp-20

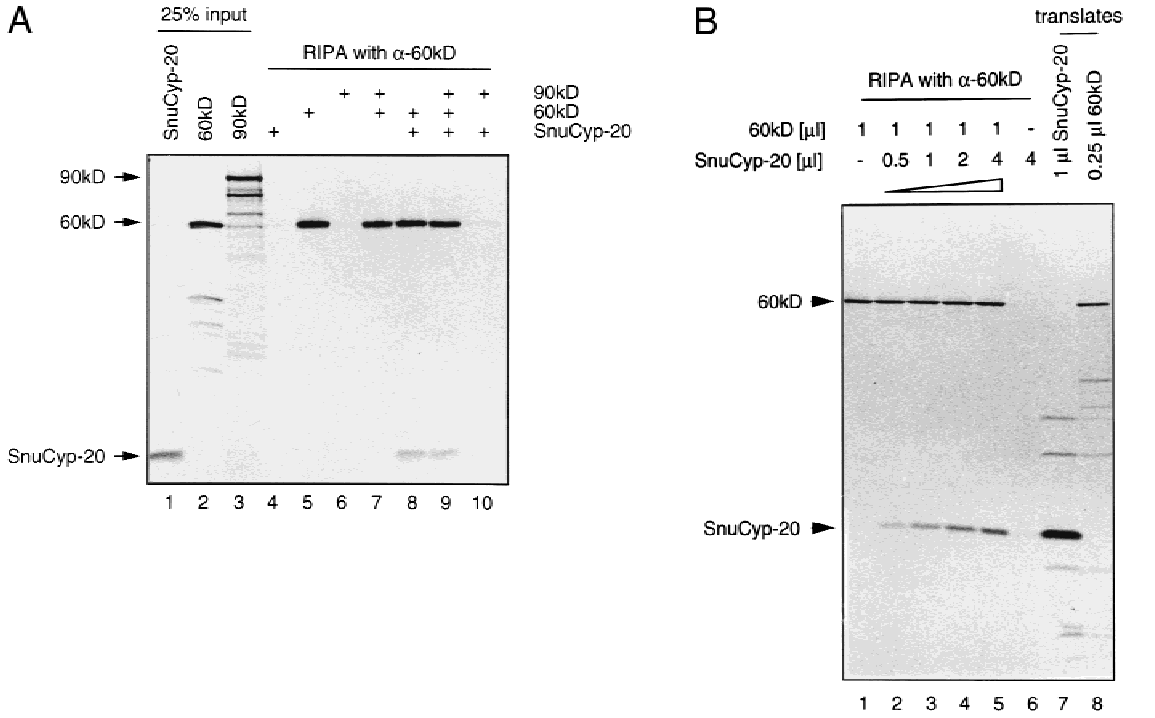


FIGURE 8. SnuCyp-20 interacts with the 60kD protein in vitro. **A:** In lanes 4–10, ^{35}S-labeled translates were incubated alone or in combination, as indicated above each lane, and subsequently precipitated with anti-60kD antibodies as described in Materials and Methods. In vitro-translated proteins shown in lanes 1–3 are equivalent to 25% of the amount used in the immunoprecipitation assays. Proteins were fractionated on a 13% SDS/polyacrylamide gel and visualized by fluorography. **B:** Constant input of 60kD translate and increasing amounts of SnuCyp-20 translate, as indicated above each lane, were coprecipitated as in A. In lane 6, SnuCyp-20 was incubated in the absence of 60kD translate. Lanes 7 and 8 show 25% of the amount of the translate used in lane 5. Proteins were fractionated on a 13% SDS/polyacrylamide gel and visualized by fluorography.

translate were incubated with constant amounts of 60kD translate, a complex containing roughly equal molar amounts of the two proteins could be precipitated (Fig. 8B, lanes 1–5). Surprisingly, despite the fact that a heteromeric complex consisting of the SnuCyp-20, 60kD, and 90kD proteins could be isolated from purified snRNPs, coimmunoprecipitation of the 90kD protein with in vitro-translated 60kD and/or SnuCyp-20 proteins was not detected (Fig. 8A, lanes 7 and 9). This result was obtained irrespective of the order in which the three proteins were mixed with each other (not shown). The formation of a stable SnuCyp-20/60kD/90kD heteromer may thus require additional factors not present in our in vitro reconstitution system.

DISCUSSION

We show that the 20kD protein of the human [U4/U6.U5] tri-snRNP complex, which we denote SnuCyp-20, is a novel human cyclophilin. SnuCyp-20 shares a strongly conserved central region with other known cyclophilins, including those amino acids that have been shown previously to be important for the binding of cyclosporin A to CypA or CypB (Mikol et al., 1993, 1994; Thériault et al., 1993). As expected for a canonical cyclophilin, SnuCyp-20 appears to exhibit peptidyl-prolyl *cis/trans* isomerase activity in vitro. The evidence for this was provided by our finding that highly purified 25S [U4/U6.U5] tri-snRNPs were active in vitro in *cis/trans* isomerizing the alanine-proline bond of a model peptide substrate (Fig. 4), whereas 20S U5 or 12S U1 and U2 snRNPs were not. In fact, when a mixture of anti-m_3G affinity-purified snRNPs were fractionated by centrifugation on a glycerol gradient, PPIase activity correlated strictly with the presence of SnuCyp-20 in the gradient fractions (Fig. 4), indicating that the PPIase activity of tri-snRNPs is due to the 20kD tri-snRNP protein (SnuCyp-20). Moreover, the isomerase activity of tri-snRNPs could be inhibited by the addition of cyclosporin A, demonstrating that the PPIase activity is exclusively due to a cyclophilin and not caused by a member of other PPIase families such as FK506 binding proteins (see Galat, 1993, for a review on PPIase families). We note that the PPIase activity of the tri-snRNP-associated SnuCyp-20 is significantly lower compared with other cyclophilins such as CypA (by 1–2 orders of magnitude; Fig. 4 and data not shown). At present, we do not know whether this reflects distinct substrate specificities of the two cyclophilins, or whether

the intrinsic catalytic activity of SnuCyp-20 is significantly lower. In this respect, it is interesting to note that the PPIase activity of *C. elegans* Cyp11, the likely functional homologue of human SnuCyp-20 (Fig. 3), is also significantly lower compared with the *C. elegans* CypA homologue (Page et al., 1996).

Immunofluorescence microscopy and biochemical fractionation of nuclear and cytoplasmic extracts revealed that the major fraction of cellular SnuCyp-20 is located in the nucleus. SnuCyp-20 colocalizes with nuclear speckles that have been identified as typical snRNP-containing structures. These results, together with our finding that SnuCyp-20 is stably associated with [U4/U6.U5] tri-snRNPs in vitro, suggest that SnuCyp-20 may function, at least in part, in close association with the [U4/U6.U5] tri-snRNP complex (see also below). Recently, two additional nuclear cyclophilin-related proteins have been described that contain, in addition to a cyclophilin domain, either an RNA-binding domain (Mi et al., 1996) or an RS (arginine/serine-rich) domain (Nestel et al., 1996; Bourquin et al., 1997). RS domains are typical features of members of the SR protein family of pre-mRNA splicing factors (reviewed by Fu, 1995). Although the RS domain containing cyclophilin, like SnuCyp-20, is also located in nuclear speckles (Bourquin et al., 1997), it is presently unclear whether it participates in pre-mRNA splicing.

Aside from a few exceptions, such as the *Drosophila* cyclophilin NinaA, the in vivo substrates of most cyclophilins are generally not known (see Introduction). The characterization of the 20kD tri-snRNP protein as a novel cyclophilin therefore raised the interesting question as to the identity of its interaction partner(s) within the tri-snRNP. Coimmunoprecipitation studies performed with in vitro-translated SnuCyp-20 and purified snRNPs revealed a striking specificity of interaction of SnuCyp-20 with [U4/U6.U5] tri-snRNPs. For example, in vitro-translated SnuCyp-20 bound exclusively to [U4/U6.U5] tri-snRNPs as opposed to purified U1, U2, or U5 snRNPs. Moreover, despite its high overall sequence similarity with SnuCyp-20 (51% identity), in vitro-translated CypA did not bind to tri-snRNPs (Fig. 6B) nor did it inhibit the association of SnuCyp-20 with tri-snRNP. The reason for the specificity of interaction of SnuCyp-20 with the [U4/U6.U5] tri-snRNP became clear when we discovered that SnuCyp-20 forms a stable complex with the U4/U6-specific 60kD and 90kD proteins, the human homologues of the yeast Prp4 and Prp3 proteins (Lauber et al., 1997). This was convincingly demonstrated by our finding that SnuCyp-20 and the 90kD protein could be coisolated together with the 60kD protein by immunoaffinity chromatography with an antibody specific for the 60kD protein (Fig. 8). The SnuCyp-20/60kD/90kD protein heteromer, as isolated from [U4/U6.U5] tri-snRNPs, is surprisingly stable. It not only withstands 0.5 M KCl, as used during immunoaffinity chromatography (Fig. 7C), but also centrifugation on a glycerol gradient containing 0.4 M NaSCN (Fig. 7AB). This suggests that both hydrophobic and ionic interactions contribute to the stability of the SnuCyp-20/60kD/90kD heteromer. Recently, a similar heteromeric protein complex containing the U4/U6-specific 60kD, 90kD, and the 20kD tri-snRNP cyclophilin was independently isolated by extensive biochemical fractionation of HeLa nuclear extracts (Horowitz et al., 1997).

With the goal of investigating protein–protein interactions in the SnuCyp-20/60kD/90kD heteromer in more detail, we performed coimmunoprecipitation experiments with each of these proteins translated in vitro. In this way, we could indeed demonstrate a direct interaction between SnuCyp-20 and the 60kD protein. Because SnuCyp-20/60kD complex formation was saturable and salt stable (Fig. 8B and data not shown), it is likely that these two proteins also interact directly with each other within the isolated SnuCyp-20/60kD/90kD heteromer. We have recently shown that the U4/U6-specific 60kD protein, like its yeast homologue Prp4p, contains seven WD-40 repeats in its C-terminal half, suggesting that it may adopt a propeller-like structure as observed for β-transducin (Lauber et al., 1997). To our knowledge, this is the first example of the formation of a stable complex between a cyclophilin and a WD-40 protein. It will be interesting to investigate in more detail which domains of the two proteins participate in complex formation and, in particular, whether the PPIase catalytic center is involved in this recognition event.

Our findings raise the interesting possibility that SnuCyp-20 may play an important role in the assembly of the 60kD and 90kD proteins into U4/U6 snRNPs. For example, SnuCyp-20 could act as a chaperone-like molecule and, due to its interaction with the 60kD protein, impart a 60kD protein conformation necessary for complex formation with the 90kD protein. In this way, SnuCyp-20 could resemble somewhat the function of *Drosophila* NinaA cyclophilin, which stably binds and thus prevents misfolding of rhodopsin (Baker et al., 1994).

Unexpectedly, it was not possible to reconstitute a stable SnuCyp-20/60kD/90kD protein heteromer with proteins synthesized in vitro using reticulocyte lysate, suggesting that this assembly process has more complex requirements that are not met by our in vitro conditions (Fig. 8). A simple explanation for the lack of complex formation could be that one or more post-translational modifications that are missing from the in vitro-synthesized proteins are required for the stable interaction of the 90kD protein. Alternatively, it may be possible that one or more factors facilitating the stable association of the 90kD protein with the 60kD and SnuCyp-20 proteins in vivo are lacking from our reconstitution system. One particularly interesting question is whether these proteins are integrated into U4/U6 snRNPs as a pre-formed SnuCyp-20/60kD/90kD het-

eromer or whether they interact sequentially with U4/U6 snRNPs, i.e., whether the stable de novo formation of a 90kD/60kD/SnuCyp-20 protein complex requires, at least transiently, RNA–protein interactions as well. An additional, presently open question is whether these proteins interact with U4 snRNPs or whether a preassembled U4/U6 snRNP is required for their integration into an snRNP particle. This is not only interesting in terms of the complex de novo biogenesis pathway of U4/U6 snRNPs, which requires cytoplasmic as well as nuclear assembly events (see Raker et al., 1996, for a review on the biosynthesis of UsnRNPs), but also when it is considered that the [U4/U6.U5] tri-snRNP appears to be recycled after each round of splicing (see Introduction). At present, little is known about how the dissociated U4, U6, and U5 snRNPs reassemble and it will be interesting to determine whether the SnuCyp-20/60kD/90kD complex also undergoes coordinated cyclic dissociation and assembly events.

As discussed in the Introduction, the spliceosome undergoes many conformational rearrangements at the RNA and protein level, and it is likely that proteins of the DEAD/DEXH-box RNA helicase family play an important role in mediating some of these conformational changes through a direct interaction with RNA. Due to its apparent PPIase activity, it is conceivable that SnuCyp-20 may also function as a molecular switch by directly triggering conformational changes in one or more spliceosomal proteins (e.g., the U4/U6-specific 60kD and 90kD proteins). These changes in turn could lead to conformational transitions in spliceosomal RNAs. Future reconstitution studies of the in vitro splicing reaction using mutagenized SnuCyp-20, together with the 60kD and 90kD proteins, should help to address some of these questions.

MATERIALS AND METHODS

Isolation of snRNPs and U4/U6-specific protein complexes

Nuclear extracts were prepared from HeLa cells (Computer Cell Culture Company, Mons, Belgium) according to Dignam et al. (1983). U1, U2, and U5 snRNPs and [U4/U6.U5] tri-snRNP particles were purified from nuclear extracts by immunoaffinity chromatography at 250 mM NaCl using the monoclonal antibody H20 as described (Laggerbauer et al., 1996). The snRNP particles were further fractionated by centrifugation on a 10–30% (w/w) glycerol gradient according to Laggerbauer et al. (1996). The RNA-free 20kD/60kD/90kD heteromeric protein complex was isolated by anti-60kD immunoaffinity chromatography as described previously (Lauber et al., 1997), with the following modifications. Initially, 25S [U4/U6.U5] tri-snRNPs were dissociated by treatment with buffer W [20 mM HEPES/KOH, pH 7.9, 1.5 mM $MgCl_2$, 0.5 mM DTE, 0.5 M PMSF, 5% (v/v) glycerol] containing 0.4 M KCl and the mixture was passed over a 1-mL anti-60kD immunoaffinity column. U4/U6 snRNPs were retained on the column due to binding of the U4/U6-specific 60kD protein to the antibodies (Lauber et al., 1997). After briefly washing the affinity column with buffer W containing 0.4 M KCl to remove unbound proteins and U5 snRNPs, the column was subsequently washed with ca. 30 bed volumes of buffer W containing 0.5 M KCl. Under these conditions, the U4/U6-specific proteins dissociate from U4/U6 snRNP cores and only the 60kD protein (and any protein stably interacting with it) remain bound to the affinity column. Anti-60kD antibody-bound proteins were subsequently eluted from the column with buffer W containing 0.5 M KCl and 50 μM of competing 60kD synthetic peptide against which the anti-60kD antibody had been raised initially (Lauber et al., 1997). The peptide eluate of the affinity column was subsequently subjected to centrifugation on a 5–20% glycerol gradient containing 20 mM HEPES/KOH, pH 7.9, 500 mM KCl, 1.5 mM $MgCl_2$. Centrifugation was performed at 4 °C for 4 h at 55,000 rpm in an SW60 rotor. After centrifugation, each gradient was fractionated manually into 0.15-mL fractions.

For the partial dissociation of [U4/U6.U5] tri-snRNPs in the presence of 0.4 M sodium thiocyanate (NaSCN), tri-snRNPs purified by glycerol gradient fractionation of anti-m_3G immunoaffinity eluates were concentrated by ultracentrifugation (265,200 $\times$ *g*, 17 h, 4 °C) and the pellet resuspended in NaSCN buffer (20 mM HEPES/KOH, pH 7.9, 1.5 mM $MgCl_2$, 400 mM NaSCN). Subsequently, this mixture was loaded onto a 5–20% (w/v) glycerol gradient containing NaSCN buffer. Centrifugation was performed at 4 °C for 21 h at 33,000 rpm in an SW40 rotor and the gradient was fractionated manually into 0.4-mL fractions.

Microsequencing and database search

Proteins were isolated from 20 mg of snRNPs by phenol extraction and acetone precipitation and were separated by preparative SDS/PAGE. Coomassie-stained bands of the 20kD protein were excised from the gel, concentrated by funnel well SDS/PAGE, and blotted onto a PVDF membrane as described previously (Lauber et al., 1996). Partial amino acid sequences of tryptic peptides of the protein were determined by microsequencing on an ABI 477A sequencer. Three peptides were obtained: GSTFHR, DFMIQGGDFVN, and KIENVPTGPNNK.

Database searches using the TBLASTN program (Altschul et al., 1990) were performed on the BLAST server at the National Center for Biotechnology Information. Eleven ESTs could be identified that code for at least two of the three peptides and form an overlapping contiguous sequence. The EST with the longest 5′ terminus (accession number T53949) was obtained commercially and sequenced in full. Its conceptual translation was used for a BLASTP homology search.

Immunization and antisera

*Bam*H I and *Sma* I restriction sites were added by PCR to the open reading frame, excluding the start methionine, of EST T53949. This fragment was subsequently subcloned into pGEX 4T-2 (Pharmacia). Overexpression of the GST-fusion protein in *Escherichia coli* DH5α was induced by incubation with isopropyl-β-D-thiogalactoside (1 mM) for 4 h at 30 °C. Overexpressed fusion protein was purified from inclusion bodies by preparative SDS/PAGE as described (Lauber et al., 1996) and used for immunization of rabbits (Harlow & Lane, 1988).

For western blot analysis, proteins were separated on a 13% polyacrylamide gel, electroblotted onto nitrocellulose, and immunostained using alkaline phosphatase-conjugated secondary antibodies as described previously (Lehmeier et al., 1990).

In vitro translation and radioimmunoprecipitation assays

For in vitro translation, the EST was excised with *Sac* I and *Xho* I, both in the multiple cloning site of the EST vector, and subcloned into pBS SK^-. The cDNA encoding CypA was subcloned from a pGEX expression vector, kindly provided by M. Tropschug, into the *Bam*H I and *Xho* I sites of pBS SK^-. Both plasmids were subsequently linearized with *Xho* I and transcribed with T3 RNA polymerase. Translation reactions were performed with 10 μg of mRNA transcript in a 75-μL assay using wheat germ extract (Promega) as described by the manufacturer. Alternatively, 1 μg of uncut plasmid DNA was used in a coupled transcription/reticulocyte translation system (Promega) as described by the supplier. Calculation of the amount of protein formed during translation is based on incorporation of 3% of the radiolabeled methionine, a value that is given by the supplier of the translation systems and agrees with our estimate obtained by measuring TCA-precipitated radiolabel.

For immunoprecipitation assays, the respective proteins or snRNP particles and translation reactions were incubated with 10 μL packed volume of pre-blocked antibody–protein A Sepharose beads in 400 μL IPP_{150} buffer (20 mM Tris/HCl, 150 mM NaCl, 0.05% NP-40) for 1 h at 4 °C with constant rotation. The beads were washed five times with 1 mL of IPP_{150} buffer and bound proteins were released with SDS and fractionated by SDS/PAGE (Hermann et al., 1995).

Peptidyl-prolyl *cis*/*trans* isomerase assay

The PPIase assay was performed as described by Fischer et al. (1984) and Harrison and Stein (1990). In short, the synthetic peptide *N*-succinyl-Ala-Ala-Pro-Phe-4-nitroanilide was incubated in the presence or absence of snRNP particles. Peptides in the *trans*-conformation were hydrolyzed after phenylalanine by chymotrypsin, and nitroaniline release was monitored spectrophotometrically at 400 nm. Assays were performed at 10 °C. Background isomerization was measured in gradient buffer containing 20% glycerol, and the PPIase activity is expressed in apparent units $[(k_{cat}/k_o) - 1]$.

Immunofluorescence microscopy

HeLa cells were grown at 37 °C (5% CO_2) to about 70% confluency on glass cover slips in Dulbecco's modified Eagle medium (GIBCO) supplemented with 10% fetal calf serum and penicillin/streptomycin. The cells were rinsed three times with phosphate buffered saline (PBS, 140 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.3), fixed for 10 min with 2% (w/v) paraformaldehyde in PBS, rinsed three times with PBS, and permeabilized with PBS/0.2% Triton-X-100 for 5 min. After washing three times with PBS, the cells were incubated for 1 h in PBS containing 1% (w/v) bovine serum albumin (PBS/BSA) to block nonspecific binding. Cells were then incubated for 1 h with the primary antibodies diluted 1:1,000 in PBS/BSA, washed three times with PBS, and incubated for 1 h with Texas Red-conjugated donkey anti-rabbit and Cy2-conjugated donkey anti-mouse secondary antibodies (Amersham) diluted 1:500 in PBS/BSA. Finally, the coverslips were washed three times with PBS, air dried, and mounted on a drop of Fluoprep (BioMerieux, France). Fluorescence images were recorded and processed using a confocal laser scanning microscope (Zeiss).

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