Solution Structure of Human Peptidyl Prolyl Isomerase-like Protein 1 and Insights into Its Interaction with SKIP*  

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The human PPIL1 (peptidyl prolyl isomerase-like protein 1) is a specific component of human 35 S U5 small nuclear ribonucleoprotein particle and 45 S activated spliceosome. It is recruited by SKIP, another essential component of 45 S activated spliceosome, into spliceosome just before the catalytic step 1. It stably associates with SKIP, which also exists in 35 S and activated spliceosome as a nuclear matrix protein. We report here the solution structure of PPIL1 determined by NMR spectroscopy. The structure of PPIL1 resembles other members of the cyclophilin family and exhibits PPIase activity. To investigate its interaction with SKIP in vitro, we identified the SKIP contact region by GST pulldown experiments and surface plasmon resonance. We provide direct evidence of PPIL1 stably associated with SKIP. The dissociation constant is 1.25 × 10^-7 M for the N-terminal peptide of SKIP-(59–129) with PPIL1. We also used chemical shift perturbation experiments to show the possible SKIP binding interface on PPIL1. These results illustrated that a novel cyclophilin-protein contact mode exists in the PPIL1-SKIP complex during activation of the spliceosome. The biological implication of this binding with spliceosome rearrangement during activation is discussed.

Pre-mRNA splicing, the removal of introns from mRNA precursors, is indispensable to the expression of most eukaryotic genes. The splicing of mRNA is catalyzed by spliceosome, a large machine formed by an ordered interaction of several small nuclear ribonucleoproteins (snRNPs), U1, U2, U5, U4/U6, and numerous other less stably associated non-snRNP splicing factors (1, 2). The formation of spliceosome goes through many intermediate stages. The stable intermediate complexes are the A, B, and C complexes. During the spliceosome maturation process, the most decisive step is the conversion from non-active complex B to the catalytically active spliceosome B*. The activated complex B* undergoes the first catalytic step of splicing and then forms complex C. Prior to the activation of the spliceosome and during the splicing process, a number of conformational rearrangements take place. Recently, human 45 S activated spliceosome (complex B*) and 35 S U5 snRNP have been isolated by immunoaffinity purification and characterized by mass spectrometry (3). Comparison of their protein components with those of other snRNP and spliceosomal complexes revealed a major change in protein composition. More than 100 proteins were identified in the 45 S activated spliceosome, 80 of which are known splicing factors. The rest are non-snRNP proteins, including protein SKIP and one peptidyl prolyl isomerase-like protein 1 (PPIL1) (2–4).

PPIL1 is a component in 45 S U5 snRNP in activated spliceosome (complex B*) and 35 S snRNP. It was believed to participate in the activation of spliceosome (5). The cDNA of PPIL1 was first cloned from human fetal brain, which encodes 166 amino acid residues. PPIL1 has 41.6% identity to human cyclophilin A (6). It belongs to a novel subfamily of cyclophilins, together with CypE in Dictyostelium discoideum and Cyp2 in Schizosaccharomyces pombe. SKIP (7) is another essential component in 45 S and 35 S U5 snRNP involved in the activation of spliceosome. Skip homologs have been identified from many diverse species, from yeast to human. It is a transcriptional coregulator that interacts with a variety of proteins (8–10). Since SKIP was found in 1998, more and more evidence has been accumulated for SKIP participation in pre-mRNA splicing (11, 12). It has been demonstrated that Ppp45p, ortholog of SKIP in Saccharomyces cerevisiae, is associated with spliceosome throughout the splicing process and is essential for pre-mRNA splicing (13). Neither SKIP nor PPIL1 is stably associated with U4/U6.U5 tri-snRNPs nor are they present in pre-spliceosomes; they are recruited before catalytic step 1 and might also function during activation of spliceosome (3, 4). In 2001, interaction was reported (14) between SnwA and CypE, which are postulated as the orthologs of SKIP and PPIL1 in D. discoideum, respectively. Nevertheless, no direct evidence shows that PPIL1 can bind to SKIP directly, and the molecular mechanism of the interaction is still unknown.

To investigate the interaction of PPIL1 with SKIP, we solved the solution structure of PPIL1 by NMR and have provided the kinetic constants by PPIase assay. GST pulldown experiments combined with surface plasmon resonance (SPR) measurements revealed the N-terminal fragment of SKIP-(59–129) could associate with PPIL1 in vitro tightly. Furthermore, we provided experimental evidence for the formation of PPIL1-cyclosporin A-nSKIP ternary complex by NMR titration and chemical shift perturbation experiments. According to the results of perturbation, we mapped the interfaces of PPIL1 in binding to cyclosporin A and SKIP. PPIL1-nSKIP exhibits a novel interaction mode in contrast with other known cyclophilin-protein interactions. The bio-
logical implication of the association in spliceosome rearrangement during activation is discussed. Our results implicate more complicated roles that spliceosomal cyclophilin may play during the activation of spliceosome.

MATERIALS AND METHODS

Expression and Purification and Isotope Labeling of PPIL1—The hPPIL1 cDNA was obtained by PCR from human CD34+ hematopoietic stem/progenitor cell cDNA library cloned in-frame into the Ndel/ Xhol sites of pET-22b (+) (Novagen). It was then transformed into the Escherichia coli expression strain BL21 (DE3). Bacteria were grown at 37 °C in Luria Bertani medium containing 100 mg/liter of ampicillin. Briefly, saturated Luria Bertani was diluted (1:300, v/v) in M9 minimal medium containing 0.05% 15NH4Cl and/or 0.25% [13C]glucose and incubated at 37 °C. Target protein expression was induced by addition of 0.8 mM isopropyl-1-thio-β-D-galactopyranoside to mid-log phase cultures (A600, 0.6–0.8). The culture was shaken at 20 °C overnight. After 20 h of additional growth, bacteria were harvested by centrifugation. The pellet was resuspended in a buffer containing 50 mM phosphate, pH 7.5, 500 mM NaCl. Cells were disrupted by sonication, and debris was spun down at 15,000 rpm, 4 °C for 0.5 h in a Beckman centrifuge using a JA-17 rotor. After 20 h of additional growth, bacteria were harvested by centrifugation. The pellet was resuspended in a buffer containing 50 mM phosphate, pH 7.5, 500 mM NaCl. Cells were disrupted by sonication, and debris was spun down at 15,000 rpm, 4 °C for 0.5 h in a Beckman centrifuge using a JA-17 rotor. The supernatant was applied to a HiTrap chelating resin (Amersham Biosciences) charged with Ni2+ ions. The column was washed with 50 mM imidazole in 50 mM phosphate buffer, pH 7.5, 500 mM NaCl, and the protein was eluted using 200 mM imidazole in the same buffer. The purity of the protein was assessed by SDS-PAGE. The final yield for 15N-labeled protein was 30 mg/liter and for 13C/15N-labeled protein was 20 mg/liter. The purity of the protein was >95%.

Characterization of Recombinant PPIL1—When recombinant PPIL1 was purified and concentrated to 50 mg/ml, only a few strong peaks could be observed in the 15N-H HSQC due to severe aggregation. The peaks on HSQC were not well dispersed, and the number of the peaks did not increase significantly until the sample was diluted in the NMR buffer to the concentration of 10 mg/ml, estimated by BCA kits (Pierce). When recombinant PPIL1 was assayed for PPIase activity essentially as described by Fischer et al. (15) with the suggested substrate solvent application as described by Kofron et al. (16). The peptide substrate N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide (Sigma) was dissolved in trifluoroethanol with 470 mM LiCl to give 25 mM stock solution. The experimental setup was as follows: PPIL1 was diluted into 50 mM HEPES, 86 mM NaCl, pH 8.0 (PPlase buffer) to give 1 μM stock. In a 1-ml cuvette, 6 μl of PPIL1 (10 nM final concentration) was added to 535 μl of PPlase buffer and incubated on ice for 1 h. Chymotrypsin (60 μl of 6 mg/ml) was added to the cuvette, followed by transfer of the cuvette to a thermostated cell holder by which the reaction temperature was maintained at 5 °C. The reaction was started by the addition of 1.2 μl of peptide substrate (50 μM) followed by rapid mixing. The absorbance at 395 nm due to the release of p-nitroanilide was recorded with data collected every 0.1 s over a 2-min period. A number of samples were tested using the above method. Cyclosporin A inhibition assays were carried out essentially as described above except that the samples were preincubated for a minimum of 60 min with varying amounts of cyclosporin A (Calbiochem).

GST Pulldown Experiments in Vitro—For interaction of His-tagged PPIL1 with GST-SKIP (1–172), GST-SKIP (1–64), GST-SKIP (59–129), and GST-SKIP (126–172), recombinant GST and GST fusion protein were immobilized on 300-μl bed volume of glutathione-agarose beads. After washing with 3 ml of GST binding buffer, the beads were incubated with an excessive amount of His-tagged PPIL1, which had been preincubated with cyclosporin A for 1 h, in 500-μl total volume at 4 °C overnight on a rotator. The beads were washed by GST binding buffer supplemented with different concentrations of NaCl (2 ml of each buffer). Finally, the beads were eluted by GST binding buffer supplemented with 10 mM glutathione. The bound proteins were detected by SDS-PAGE and stained with Coomasie Brilliant Blue. The bound PPIL1 were further affirmed by immunoblot using His tag antibody.

Surface Plasmon Resonance Measurements—SPR experiments were carried out. nSKIP (1–172) was coupled to a carboxymethyl-dextran CMS sensor chip with an amine coupling kit. Binding was observed upon injection of different concentrations of PPIL1. The plateau values reached after completion of the association reactions were analyzed by a Langmuir binding isotherm.

NMR Spectroscopy—The NMR sample of 15N/13C, 15N-PPIL1 was prepared and NMR experiments were recorded for assignments as described (17). NOE distance restraints were generated using 15N-edited NOESY and 13C-edited NOESY spectra employing 130-ms mixing times. NMR data were processed by NMRPipe and NMRDraw software (18) and assigned with Sparky (19). The CSI (20) program was used to obtain the backbone dihedral angles (φ and ψ) in secondary structures on the basis of chemical shift information. The NOE-derived distance restraints were classified into four groups with the upper boundaries of 3.0, 4.0, 5.0, 6.0 Å and lower boundary of 1.80 Å on the basis of NOE intensity measurements. Hydrogen bond restraints were defined from slow exchanging amide protons identified after exchange of the H2O buffer to D2O.

Structure Calculations—Structure calculation for PPIL1 was performed on the basis of proton-proton NOE restraints and dihedral angle restraints (φ and ψ) with a simulated annealing protocol using the CNS v1.1 program (21). High temperature torsion angle dynamics was performed at 50,000 K for 15 ps (1,000 steps) followed by a 15-ps cooling phase. Initial structure calculations included only hydrogen bonds in defined secondary structures from CSI. In the following refinement calculation, only hydrogen bonds whose donors could be identified unam-

PPlase Activity Assay and Cyclosporin A Inhibition Assays—Purified recombinant PPIL1 was assayed for PPlase activity essentially as described by Fischer et al. (15) with the suggested substrate solvent application as described by Kofron et al. (16). The peptide substrate N-succinyl-Ala-Ala-Pro-Phe-p-nitroanilide (Sigma) was dissolved in trifluoroethanol with 470 mM LiCl to give 25 mM stock solution. The experimental setup was as follows: PPIL1 was diluted into 50 mM HEPES, 86 mM NaCl, pH 8.0 (PPlase buffer) to give 1 μM stock. In a 1-ml cuvette, 6 μl of PPIL1 (10 nM final concentration) was added to 535 μl of PPlase buffer and incubated on ice for 1 h. Chymotrypsin (60 μl of 6 mg/ml) was added to the cuvette, followed by transfer of the cuvette to a thermostated cell holder by which the reaction temperature was maintained at 5 °C. The reaction was started by the addition of 1.2 μl of peptide substrate (50 μM) followed by rapid mixing. The absorbance at 395 nm due to the release of p-nitroanilide was recorded with data collected every 0.1 s over a 2-min period. A number of samples were tested using the above method. Cyclosporin A inhibition assays were carried out essentially as described above except that the samples were preincubated for a minimum of 60 min with varying amounts of cyclosporin A (Calbiochem).
biguously were added. In the final calculations, an ensemble of 100 structures (no distance violations >0.3 Å and no dihedral angle violations >5°) was generated from unambiguous NOEs previously determined. 20 models were selected on the basis of energetic criteria (low total energy, using the accept.inp routine) to form a representative ensemble of the calculated structures.

NMR Backbone Relaxation Experiments—\(^{15}N\) relaxation experiments were carried out at 295 K on a Bruker DMX500 NMR spectrometer. \(^{15}N\) relaxation measurements were carried out using the published methods (22). \(^{15}N\) \(T_1\) relaxation rates were measured with eight relaxation delays: 11, 62, 142, 243, 364, 525, 757, and 1150 ms. \(^{15}N\) \(T_2\) relaxation rates were measured with six relaxation delays: 17.6, 35.2, 52.8, 70.4, 105.6, and 140.8 ms. A recycle delay of 1 s was used for measurement of \(T_1\) and \(T_2\) relaxation rates. The spectra measuring \(^1H\)–\(^{15}N\) NOE were acquired with a 2-s relaxation delay followed by a 3-s period of proton saturation. The spectra recorded in the absence of proton saturation employed a relaxation delay of 5 s. The exponential curve fitting and extract of \(T_1\) and \(T_2\) were processed by Sparky. The relaxation data \(R_1/T_1, R_2/T_2\), and \(\eta\) were analyzed by Fast ModelFree v1.0.

Chemical Shift Perturbation—For detecting the cyclosporin A and nSKIP binding sites on PPIL1, 1 mM \(^{15}N\)-labeled PPIL1 was used. After \(^1H\), \(^{15}N\)-HSQC spectrum of free PPIL1 was recorded, and the sample was titrated with cyclosporin A in the method described by Weber et al. (23). After the \(^{15}N\)-\(^1H\) HSQC of PPIL1-cyclosporin A was recorded, the sample was further titrated with 1 mM unlabeled SKIP until there was no change in the spectrum recorded. The final concentrations of both proteins at the end of the titration were ~0.5 mM. All HSQC spectra for mapping the cyclosporin A and nSKIP binding interfaces on PPIL1 were performed on Bruker 600 MHz at 295 K.

Exchange Experiments of PPIL1-nSKIP Complex in D_2O—Freeze-dried \(^{15}N\)-PPIL1 mixed with nSKIP-(59–129) were dissolved in D_2O. \(^{15}N\)-\(^1H\) HSQC spectra were recorded to identify slow exchanging HNs of \(^{15}N\)-PPIL1 in the complex.

RESULTS

Peptidyl Prolyl Cis-trans Isomerase Activity and Its Inhibition Activity by Cyclosporin A—PPIL1 clearly accelerates the rate of isomerization of the tetrapeptide substrate relative to the uncatalyzed thermal isomerization rate, and the catalysis is inhibited by addition of the cyclophilin binding drug cyclosporin A (in supplemental data). The enzyme reaction was found to follow Michaelis-Menten kinetics with the velocity of the reaction (\(v\)) increasing with substrate concentration [S] (Fig. 1). First-order rate kinetics were observed, and a double reciprocal Lineeweaver-Burke plot of 1/\(v\) against 1/[S] gave values of \(k_{cat}/K_m\) 960 s\(^{-1}\), \(K_m\) 230 \(\mu\)M, which corresponds to a value of \(k_{cat}/K_m\) of \(4.2 \times 10^6 \text{M}^{-1} \text{s}^{-1}\). These values are similar to the published values for human CypA, which has \(k_{cat} 12700 \text{s}^{-1}\), \(K_m 870 \mu\)M and \(k_{cat}/K_m 14.6 \times 10^6 \text{M}^{-1} \text{s}^{-1}\) (16).

Structure Determination—The solution structure of the recombinant protein PPIL1 was determined by multidimensional heteronuclear NMR spectroscopy. The last 8 amino acids as artifact from vector were not included in structure calculations. Fig. 2 shows an ensemble of 20 NMR structures and a ribbon representation of the energy-minimized average structure of human PPIL1 by MOLMOL (24). The coordinates of these 20 NMR structures have been deposited into the Protein Data Bank (code 1XWN). Table 1 lists the structural statistics for the 20 deposited NMR structures. The root mean square deviation of the well defined secondary structure regions of the 20 structures to the average structure is 0.58 Å for the backbone and 1.08 Å for the heavy atoms. In contrast, the N-terminal residues (amino acids 1–11) are disordered because of few medium- and long-range NOEs.

A PROCHECK (25) analysis of the 20 NMR structures indicated that >95% of the residues lie in the most favored region and additional allowed region of the Ramachandran plot. The residues in the disallowed regions were those in the terminal part or in the loops because of the paucity of inter-residual NOEs.

Description of PPIL1 Structure—Fig. 2A shows the superimposition of the backbone (N, Ca, C') of the best fit of 20 structures with the lowest energy. The overall architecture of PPIL1 is similar to that of
TABLE 1
Summary of structure statistics

| Distance restraints | 544 | 551 |
|---------------------|-----|-----|
| Sequential (|i–j| = 1) | Medium range (2 < |i–j| < 4) | Long range (|i–j| > 5) |
| Hydrogen bonds | 697 | 96 |
| Total | 2205 |

| Dihedral angle restraints | 59 | 59 |
|---------------------------|----|----|
| Mean r.m.s.d. from the experimental restraints | 0.0037 ± 0.0009 | 0.1010 ± 0.0193 |
| Mean r.m.s.d. from idealized covalent geometry | 0.0011 ± 0.00005 |
| Bond | 0.2752 ± 0.0027 |
| Angle | 0.1084 ± 0.0057 |
| Improper | 0.0057 |
| Mean energies (kcal mol⁻¹) | 585.79 ± 15.35 |
| E-total | 566.42 ± 10.42 |
| E-bond | 21.51 ± 2.20 |
| E- improper | 6.66 ± 0.58 |

| PROCHECK Ramachandran Plot analysis (%) | 71.3% |
|----------------------------------------|------|
| Residues in most favored regions | 24.3% |
| Residues in additionally allowed regions | 3.6% |
| Residues in generously allowed regions | 0.8% |

| Structural r.m.s.d. for secondary structures regions (Å) | 0.58 |
|----------------------------------------------------------|-----|
| Backbone heavy atom (N, Cα, and C') | Heavy atom |
| 0.08 |

The Φ and ψ angle restraints are generated from secondary structures by CSL.

Mean r.m.s.d., root mean square deviation.

All non-Gly residues, Φ/ψ of most favored, and additional allowed regions are given by Procheck (23).

Atoms of well defined secondary structure regions: residues 13–64, 96–101, 110–115, and 157–164.

After ¹H-²H exchange experiments, we found most observable HNs exist in regular secondary structures. However, it is interesting that no backbone amide protons in helix α2 show slow exchange, which indicates helix α2 may not be in very rigid conformation or it may be exposed to the solvent. However, the NαH2 of Asn-140 exchanged slow as observed in the recorded HSQC in D2O, which probably H-bonded to O’ of Gly-137. In the solution structure of PPIL1, helix α2 was further stabilized by a network of hydrophobic interactions between well conserved amino acids such as Met-20, Ile-56, Ile-62, Val-114, Leu-116, Val-139, Val-142, and Val-145 (in supplemental data). Residue 55–57 in the β3 strand showed fast amide proton exchange in contrast to the residues in the other β strands, which have been described as “structure breath” in the solution structure of CypA (27). This could be explained as the potential ability of adjusting the interior side chains to fit the binding of its substrates or inhibitors for two enzyme activity sites (Arg-55, Ile-57) located on β3.

The Structure Comparison of PPIL1 and Cyclophilin A—In Fig. 3A, PPIL1 is superimposed upon the structure of human cyclophilin A. The root mean square deviation for most defined secondary structures of Ca atoms of PPIL1 and CypA is 1.2 Å. PPIL1 is 41.6% identical to CypA (at the amino acid level). Fig. 4 shows the sequence alignment of PPIL1 with several human cyclophilins. The PPIL1 structure is highly conserved when compared with the structure of human cyclophilin A, the best characterized member of the family (26, 27). The active sites of PPIL1 are also very similar to those found in human CypA; all 13 residues important in binding cyclosporin A are conserved. This is consistent with the facts that PPIL1 exhibits PPIase activity and the activity could be inhibited by cyclosporin A. By comparing the two structures, we found there were two highly conserved backbone-backbone hydrogen bonds located outside of regular secondary structures: Ala-32 NH–O' Tyr-28 is in a helix-like turn, and Ser-51 NH–O' Tyr-48 is a part of a β-turn (in supplemental data).

Despite the similarity in their overall structures, they are quite different at the C-terminal of helix α1 because of the loss of 3 amino acids in the loop linking α1 and β3 in PPIL1. In human cyclophilins, this feature is only present in high molecular mass cyclophilins Cyp-60 and Cyp-73 (29). A special turn occurs following α1 due to the loss of inserted amino acids, and the conserved β-turn is linked to α1 by two hydrogen bonds, Tyr-48 NH–O' Leu-42 and Tyr-47 NH–O' Leu-42 (in supplemental data). The special hydrogen net-
work makes the C-terminal of helix α1 of PPIL1 significantly different from that of hCypA and close to the loop G65-Y78 in structure comparison. The loop following α1 (65–74) is in poorly defined conformation because of the lack of inter-residual NOEs. We also note that there is one amino acid insert in the loop preceding α8, which causes the displacement of residue 146–151 with respect to the corresponding residues in hCypA; this leads to a significantly altered conformation of this loop with respect to hCypA. The respective loops in other cyclophilins (30, 31) resemble, however, more of the loop in PPIL1 than of hCypA.

N-terminal Fragments of SKIP Bind to PPIL1 Tightly—Although interaction of SnwA and CypE, orthologs of PPIL1 and SKIP, has been reported by yeast two-hybrid experiment (14), the PPIL1 binding region in SKIP is still unknown and no evidence shows direct interaction. To determine the binding region of SKIP with PPIL1, we did GST pulldown experiments to investigate the interaction of GST-SKIP172 with recombinant PPIL1 in vitro. The results indicate that only two fragments of SKIP, 1–172 and 59–129, bind PPIL1 with high affinity (Fig. 5).

As described above, nSKIP-(59–129) is sufficient to associate with PPIL1. Surface plasmon resonance measurements were carried out to study the interaction between nSKIP-(59–129) and PPIL1 (in supplemental data). The dissociation constant of 1.25 × 10^{-7} M was obtained. These results reveal that PPIL1 can interact tightly with nSKIP-(59–129) in vitro.

Formation of PPIL1-Cyclosporin A-nSKIP Ternary Complex—To investigate the details of interaction between cyclosporin A and PPIL1, we used an NMR chemical shift perturbation method (in supplemental data) in which a series of 15N-1H HSQC of PPIL1 was recorded with successive addition of cyclosporin A. Upon cyclosporin A binding, resonances of most peaks of unbound PPIL1 weakened continuously and new resonances appeared with significant chemical shift differences. This indicates that slow exchange exists for cyclosporin A binding on the NMR time scale. The statistics of chemical shift change peaks on 15N-1H HSQC help to map the cyclosporin A binding interface (Fig. 6B). We found that most of the residues involved in binding to cyclosporin A were conserved in the reported cyclophilin A-cyclosporin A complex (32). Several key residues (Arg-55, Gln-63, Trp-121, and His-126) that were H-bonded to backbone O of cyclosporin A also exist in PPIL1 (Fig. 3B). After superimposing PPIL1 to cyclophilin A, we found that side chains of these residues share a similar orientation and facilitate the formation of PPIL1-cyclosporin A complex. Unexpectedly, we found the peak of HN of Trp-121 finally disappeared, which exhibited intermediate exchange. In comparison with the cyclophilin A-cy-
cyclosporin A complex, the hydrogen bond linked to HN of Trp-121 was probably weaker in the PPIL1-cyclosporin complex. Probably it is because Pro-118 of PPIL1, which is a lysine in the corresponding location of cyclophilin A, makes the following 3_10 helix (Gln-120-Trp-121-Leu-122) shift slightly. As shown in Fig. 3B, the side chain of Trp-121 of PPIL1 deviates from its counterpart of cyclophilin A, which may have some negative effect on the formation of the intermolecular hydrogen bond.

We also found peaks of Thr-70 and Gly-71 changed significantly during the titration. Both residues were distant from cyclosporin A binding sites. It is possible that the chemical shift perturbation of both residues may result from indirect effects of the internal flexibility of this region.

After the formation of PPIL1-cyclosporin, another series of HSQC spectra were recorded with further addition of purified nSKIP-(59–129). The PPIL1-cyclosporine-nSKIP-(59–129) ternary complex also showed slow exchange during titration. The results revealed that the nSKIP bound to SKIP tightly, which was consistent with the dissociation constant of ~10^{-7} M. We did additional chemical shift perturbation experiments to investigate interaction of PPIL1 and SKIP without cyclosporin A. Chemical shifts of PPIL1 and PPIL1-cyclosporin undergo similar changes after the addition of nSKIP (in supplemental data). We concluded that binding of SKIP to PPIL1 was not affected by cyclosporin A. Furthermore, we mapped the nSKIP binding interface on PPIL1 according to statistics of the latter perturbation experiments without cyclosporin A (Fig. 6A). We found that the residues involved in binding to nSKIP-(59–129) were located at the C-terminal of β2, the loop preceding β5, and the C-terminal of β7.

H/D exchange experiments of PPIL1 in the PPIL1-nSKIP complex were done to investigate the interaction of PPIL1 with SKIP. In comparison with free PPIL1, we found HNs of Leu-27, Leu-98, and Phe-129 disappeared, whereas peaks of Lys-91 and Arg-141 showed slow exchange in the complex.

Residues in the loop region are probably directly involved in the interaction with nSKIP for the ultra-large changes of chemical shifts. The significant chemical shift change of Ile-128 and Tyr-28 may be involved in hydrophobic interaction with nSKIP-(59–129) because the methyl of Ile-128 and ring of Tyr-28 project to the surface of PPIL1. As shown in Fig. 7, the side chain of Trp-29 points to the surface of the molecule and may assist in binding nSKIP. Changes of the three hydrogen bonds
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Dynamic Properties of PPIL1—The dynamic properties of PPIL1 were probed by measuring $^{15}$N relaxation parameters. Longitudinal $T_1$ and transversal $T_2$ relaxation time as well as $^1$H–$^{15}$N heteronuclear NOE values were obtained for a total of 138 backbone amide protons (89% of all possible) (in supplemental data). No data are presented for residues for which assignments are missing or for those with significant overlap in the $^1$H–$^{15}$N HSQC spectrum. The average $^{15}$N–$^1$H NOE value of 0.81 ($\pm 0.06$) indicates that most regions of PPIL1 are relatively rigid, which is consistent with the narrow distribution of conformers in the calculated ensemble. Some residues show $^{15}$N–$^1$H NOE values above the theoretical maximum of 0.834, which may be due to chemical exchange with the solvent, provided the errors of these values are not underestimated.

A global $\tau_m$ of 13.48 ns was given by Fast ModelFree v1.0, whereas the generalized order parameters $S^2$ obtained for the 136 remaining residues. Internal mobility is restricted ($S^2 > 0.8$) for residues located in $\alpha_2$ and all eight $\beta$ strands. There are several residues with much smaller order parameters ($S^2 < 0.7$), including Gly-46, Asp-66, Gly-74, Ala-75, Ser-76, Tyr-78, His-87, Asn-108, and Gly-109. All of them are located on loops or turns linking secondary structures. The conformation of the loop region following $\beta_4$ is poorly defined in solution, perhaps because of its dynamic property. The flexibility of Asn-108, Gly-109 linking $\beta_5/\beta_6$ is well understood as both residues are involved in binding cyclosporin A.

Looking at the nSKIP binding interface on PPIL1, we found that His-87 exhibits an internal motion with a $\tau_m$ of 16.2 ps, a significantly high $T_2$ (87.64 ms), and a low $\eta$ (0.634). The time scale of these dynamic processes includes contributions on the nanosecond time scale, as well as on the millisecond time scale as evidenced by the chemical shift exchange contribution ($R_\infty$) present throughout this region (His-87-Gly-96). These results implicated that this region of PPIL1 is internal flexible, which may be required for free PPIL1 to bind SKIP.

Besides the residues discussed, the region including the C-terminal of helix $\alpha_1$ (residues Ala-43–Asn-49) undergoes some significant flexibility, with an average of $R_\infty$ 4.027 s$^{-1}$. Several positive charged amino acids (Arg-44, Arg-45, Asn-49) in this region implicated that it may exist as another protein–protein interaction interface.

DISCUSSION

There are 19 cyclophilins encoded in the human genome (33). Among them, several cyclophilins have been found as components of spliceosome. However, little is known about their function and potential binding interface with other proteins. Human PPIL1 and SKIP are both components in 45 S snRNP in activated spliceosome and in 35 S U5 snRNP and are believed to be involved in the activation of spliceosome (3, 4).

In this report, we have presented the solution structure of PPIL1 by NMR spectroscopy and provided the kinetic constants for its PPIase activity. The solution structure of PPIL1 is similar to those of other human cyclophilins and exhibits PPIase activity, which could be inhibited by potent inhibitor cyclosporin A. Results from GST pulldown, SPR, and NMR chemical shift perturbation experiments provide solid evidences that nSKIP-(59–129) could associate with PPIL1. Furthermore, we mapped cyclosporin A and nSKIP-(59–129) binding interfaces on PPIL1 by chemical shift perturbation. From structure comparison of PPIL1 with cyclophilin A and chemical shift perturbation, we found PPIL1-cyclosporin A is a typical cyclophilin-cyclophilin A interaction mode, whereas interaction of PPIL1 with nSKIP reveals a novel cyclophilin–protein interaction mode. The for-

(Leu-27 NH-O’ Pro-12, Leu-98 NH-O’ Gly-130, and Phe-129 NH-O’ Leu-98) indicate that the intramolecular interactions between $\beta_1$ and $\beta_2$, $\beta_5$ and $\beta_7$ were weakened somewhat during the formation of the complex. These shifts in the secondary structures reflect the conformational change required for the residues to participate in binding SKIP (Fig. 7).

Chemical shifts of Asp-89 and Lys-91 in the loop preceding $\beta_5$ also changed significantly during the titration. Because no appropriate H-bond donor exists, an intermolecular H-bond probably forms between HN of Lys-91 and an unknown atom of nSKIP. Asp-89 is highly conserved in orthologs of PPIL1. Presumably, it may play an important role in electronic-static interaction with nSKIP.

Chemical shift changes of some residues may not result directly from the binding ligand. Peaks of Gly-137 and Gly-143 in $\alpha_2$ helix change significantly, which could be explained by the ubiquitous interaction between $\alpha_2$ and other secondary structures and the sensitivity of glycines in contrast to other residues. In free PPIL1, the $\alpha_2$ helix is not as rigid as other secondary structures, which has been shown in $^1$H–$^2$H exchange experiments of unbound PPIL1. However, in the complex of PPIL1-nSKIP, we found a hydrogen bond (Arg-141 NH-O’ Gly-137) strengthened in $\alpha_2$ helix (Fig. 7). Although both residues changed during the titration, the peak of Arg-141 changed less than that of Gly-137. It may be that glycines more easily make some accommodation to the overall conformational changes of PPIL1 in binding nSKIP.

By rotating the nSKIP binding interface on PPIL1 by 120° counterclockwise, we find the cyclosporin A binding interface (Fig. 6). The results implicated that PPIL1 could bind cyclosporin A and nSKIP on different interfaces simultaneously, which is consistent with the formation of the PPIL1-cyclosporin A-nSKIP ternary complex.

FIGURE 7. Structure feature of potential SKIP binding sites on PPIL1 according to chemical shift perturbation experiments. Side chains of non-glycine residues that may be involved in binding SKIP are colored in yellow sticks. Several secondary structure segments ($\beta_1$, $\beta_2$, $\beta_5$, $\beta_6$, $\beta_7$, and $\alpha_2$) of PPIL1 (green) are shown for clarity. The three hydrogen bonds weakened in the PPIL1-SKIP complex are labeled in red. One strengthened hydrogen bond in the $\alpha_2$ helix in the complex is labeled in red.
formation of ternary complex supports the hypothesis that SKIP does not occupy the enzyme active sites of PPIL1 and the binding activity is not affected by cyclosporin A (5). We also assayed the PPILase activity of PPIL1-SKIP complex, which could catalyze the isomerization of substrate as free PPIL1 did (supplemental material).

The nSKIP binding interface on PPIL1 mainly consists of β2, β7, and the loop preceding ββ by NMR chemical shift perturbation experiments (Fig. 7). Interestingly, the H/D exchange experiments of PPIL1 in the bound state indicated this region probably undergoes some conformational change. Several hydrogen bonds were weakened and a new intermolecular H-bond was generated in this process. According to these facts, we hypothesized that these changes were required to facilitate key residues in this region to contact nSKIP. In addition, the chemical shift changes of the residues possibly result from indirect effects. During the formation of PPIL1-nSKIP, we found a hydrogen bond was strengthened in the α2 helix. This may result in the large chemical shift change of Gly-137, which is not within the binding region. It is interesting that although the α2 helix probably is not affected by nSKIP directly, it may remodel itself somewhat to alleviate the local conformational change by the complicated interactions with other secondary structures of PPIL1.

Detailed analysis of 15N backbone relaxation experiments indicated the region involved in nSKIP binding exhibits significant conformational or chemical exchange evidenced by R_ex. The internal flexibility of the region in free PPIL1 reflects its potential of binding ability for specific partners, such as SKIP.

The crystal structure of CypH, another spliceosomal cyclophilin in U4/U6, in complex with a peptide from U4/U6-associated protein has been reported (34). We note that the nSKIP binding interface on PPIL1 is different from the U4/U6 binding interface on CypH. Therefore, the nSKIP binding interface on PPIL1 reflects, unambiguously, that a novel contact mode exists in cyclophilins.

It has been reported that SKIP is recruited to the spliceosome before the first catalytic step of splicing and remains bound through both catalytic steps. PPIL1 was also present in 45 S activated spliceosome but was not present in the B4U1 stage just prior to its activation (4), implying that PPIL1 may play a major role in the activation of the spliceosome rather than in the stable integration of the U4/U6.U5 tri-snRNP. Moreover, PPIL1-SKIP ultimately associates with spliced-out intron after splicing of pre-mRNA as components of 35 S U5 snRNP (3).

A rearrangement occurs in the spliceosome B-to-B* transition that requires extensive conformational transition to take place. During this process, PPIL1 is recruited by SKIP. It may play an important role as a foldase or as a molecular chaperone. The residues involved in substrate binding are highly conserved in the PPIL1 structure, so PPIL1, as other cyclophilins, may have a similar preference in binding proline-rich sequences (35) in unconstrained regions in proteins in 45 S activated spliceosome.

There are two or more protein-protein interaction interfaces existing in most spliceosomal proteins with the ability to bind different partners simultaneously. Our results indicated that the N-terminal fragment of SKIP is sufficient to bind PPIL1. Besides PPIL1, SKIP has several other partners in spliceosome (5), so it may have several interfaces to recruit different effectors. The N-terminal of SKIP probably could recruit PPIL1 independently. There are also several cyclophilins that participate in assembly and activation of spliceosome at different stages. We believe each spliceosomal cyclophilin may require a specific partner to recruit it into the appropriate site in spliceosome. Thus, cyclophilins probably play more complicated roles in spliceosome than expected.

Recently it has been reported that the elevated expression of PPIL1 may play an important role in the proliferation of cancer cells and the interaction between PPIL1 and SNW1/SKIP may uncover the role of elevated PPIL1 in cancer cells (36). Although further investigation of the function of PPIL1 will be necessary, detailed structural information on PPIL1 may provide useful targets for the design of new drugs.

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