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A cyclophilin functions in pre-mRNA splicing

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We report that the cyclophilin USA-CyP is part of distinct complexes with two spliceosomal proteins and is involved in both steps of pre-mRNA splicing. The splicing factors hPrp18 and hPrp4 have a short region of homology that defines a high affinity binding site for USA-CyP in each protein. USA-CyP forms separate, stable complexes with hPrp18 and hPrp4 in which the active site of the cyclophilin is exposed. The cyclophilin inhibitor cyclosporin A slows pre-mRNA splicing *in vitro*, and we show that its inhibition of the second step of splicing is caused by blocking the action of USA-CyP within its complex with hPrp18. Cyclosporin A also slows splicing *in vivo*, and we show that this slowing results specifically from inhibition of USA-CyP. Our results lead to a model in which USA-CyP is carried into the spliceosome in complexes with hPrp4 and hPrp18, and USA-CyP acts during splicing within these complexes. These results provide an example of the function of a cyclophilin in a complex process and provide insight into the mechanisms of action of cyclophilins.

Keywords: cyclophilin/cyclosporin A/hPrp4/hPrp18/pre-mRNA splicing

Introduction

Pre-mRNA splicing takes place within a dynamic ribonucleoprotein particle termed the spliceosome, which consists of five small nuclear RNAs (snRNAs) and at least 50 proteins (Burge *et al*., 1999). Splicing occurs in two sequential reactions; however, the assembly of the spliceosome, identification of the splice sites and proper joining of the exons proceed in an ordered pathway that requires many conformational rearrangements (Staley and Guthrie, 1998). Proteins that alter the conformations and interactions of the pre-mRNA and the snRNAs have been characterized, but little is known about how the interactions among the proteins change during splicing.

The splicing factors, Prp3, Prp4 and Prp18, were identified originally in *Saccharomyces cerevisiae*. Their human counterparts, hPrp3, hPrp4 and hPrp18, are related in both sequence and function. Prp18 is needed specifically during the second catalytic reaction of splicing, in which the mRNA is formed from the splicing intermediates (Horowitz and Abelson, 1993; Horowitz and Krainer, 1997), and is a part of a network of interacting factors involved in the second step (Umen and Guthrie, 1995). Prp4 is an integral part of the U4/U6 snRNP and is required during the assembly of spliceosomes prior to the first step of splicing (Ayadi *et al*., 1997; Lauber *et al*., 1997). Prp4 contains a WD-repeat domain, which is a scaffold for the binding of the Prp3 protein (Ayadi *et al*., 1998). In humans, hPrp4 and hPrp3 form a very stable complex together with a third protein, the cyclophilin USA-CyP (also called SnuCyp-20) (Horowitz *et al*., 1997; Wang *et al*., 1997; Teigelkamp *et al*., 1998). USA-CyP is a 177 amino acid protein that is closely related to other cyclophilins in sequence and structure (Horowitz *et al*., 1997; Teigelkamp *et al*., 1998; Reidt *et al*., 2000). However, the function of USA-CyP has not been determined.

Cyclophilins are an evolutionarily conserved family of proteins found in abundance in every organism (Marks, 1996; Hunter, 1998). The ~180 amino acids that form the core of a cyclophilin fold into a single, compact domain, which contains the highly conserved 110 amino acid catalytic center. The first cyclophilin was identified based on its affinity for the immunosuppressive drug cyclosporin A (CsA). Cyclophilins subsequently were found to catalyze the *cis–trans* isomerization of peptide bonds preceding proline (Galat and Rivière, 1998). The cellular roles of cyclophilins have been difficult to establish (Marks, 1996; Hunter, 1998; Schiene and Fischer, 2000). Acting as prolyl isomerases, cyclophilins could play a role in protein folding or could act specifically to alter protein conformations. Alternatively, cyclophilins may act primarily as chaperones, binding to peptide sequences containing proline (Schreiber and Crabtree, 1992; Luban, 1996; Ivery, 2000). A limited number of examples supports each point of view. Here we show that USA-CyP functions in pre-mRNA splicing.

Results

Homology between hPrp4 and hPrp18

hPrp4 and hPrp18 have a 31 amino acid region of homology that was noted originally as the likely basis for the cross-reactivity of antibodies against hPrp18 with hPrp4 (Horowitz *et al*., 1997). These regions of hPrp4 and hPrp18 are conserved in a wide variety of organisms (Figure 1). Although some residues are conserved specifically in either the Prp4 or the Prp18 proteins, most of the conserved residues are found in both the Prp4s and the Prp18s, and a compelling alignment of the two regions can be made. The 31 amino acid homologous region spans positions 107–137 in hPrp4, 80 amino acids N-terminal to the first of its WD-repeat elements. The Prp4 proteins are homologous along their entire lengths, and the homology

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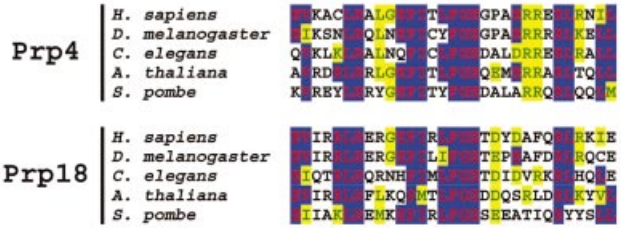

Fig. 1. Alignment of homologous regions of the Prp4 and Prp18 protein families. At positions at which at least seven out of 10 amino acids are identical or similar, the consensus residue is shown red on blue, and similar residues are shown green on yellow. Positions at which at least five amino acids are identical or six are similar are shown green on yellow. Similarities used are E~D, R~K, L~I~V~M.

of the 31 amino acid stretch is typical of that found throughout the proteins (Horowitz *et al*., 1997; Lauber *et al*., 1997; Wang *et al*., 1997). In contrast, in hPrp18, the block of homology, which begins at amino acid 83 (out of 342), occurs within a 150 amino acid region that is not otherwise evolutionarily conserved. The Prp18 proteins are strongly homologous in their C-terminal halves (Horowitz and Krainer, 1997; Jiang *et al*., 2000).

The 31 amino acid peptide appears to be found exclusively in the Prp4s and Prp18s. BLAST searches of the databases using the peptides shown in Figure 1 return only Prp4 and Prp18 homologs.

The homologous region defines a binding site for USA-CyP

We surmised that the region conserved between the Prp4s and Prp18s was a binding site for another splicing factor. USA-CyP was a good candidate for this binding because hPrp4 interacts with USA-CyP in the complex with hPrp3 (Horowitz *et al*., 1997; Teigelkamp *et al*., 1998). Furthermore, *S.cerevisiae* lacks a homolog of USA-CyP as well as the potential binding sites for USA-CyP in its Prp4 and Prp18 proteins.

We first tested whether USA-CyP forms a complex with hPrp18. USA-CyP was produced in *Escherichia coli*; gel filtration showed that USA-CyP was entirely monomeric. Purified His-tagged hPrp18 was mixed with an ammonium sulfate fraction of proteins from *E.coli* that overproduced USA-CyP; the mixture was passed over an Ni-NTA–agarose column, and the bound proteins were eluted with imidazole (Figure 2A). Both His-hPrp18 and USA-CyP bound to the resin and were eluted in ~1:1 stoichiometry (Figure 2A, lane 7). Binding of USA-CyP to the column depended on the presence of His-hPrp18 (Figure 2A, lanes 8–14). The results show that USA-CyP and hPrp18 do form a complex. The USA-CyP–hPrp18 complex was stable and could be purified in 1 M NaCl. Gel filtration chromatography showed that the complex is a heterodimer.

Formation of a complex between USA-CyP and hPrp18 suggested that USA-CyP interacts with the homologous regions of hPrp4 and hPrp18. We examined the binding of USA-CyP to peptides from hPrp4 and hPrp18. Two 31 amino acid peptides (H4 and H18) whose sequences were derived from those of hPrp4 and hPrp18 shown in Figure 1 were synthesized and covalently bound to agarose columns. The ammonium sulfate-fractionated extract from

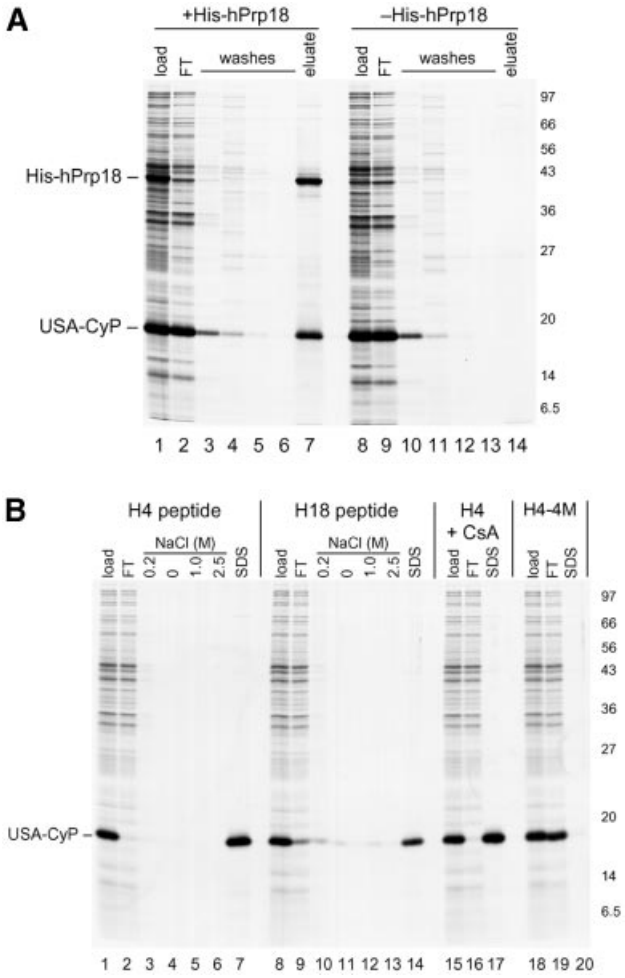

Fig. 2. Complexes of USA-CyP. (**A**) USA-CyP forms a stable complex with hPrp18. His-tagged hPrp18 (His-hPrp18) plus ammonium sulfate-fractionated extract from *E.coli* that produce USA-CyP (lane 1), flow-through from the Ni-NTA column (lane 2) and washes (lanes 3–6; washes 4–6 contained 20 mM imidazole). Proteins were eluted from the column with 400 mM imidazole (lane 7). Lanes 8–14 show the parallel experiment in the absence of His-hPrp18. (**B**) Complexes of USA-CyP with peptides from hPrp4 and hPrp18. Ammonium sulfate-fractionated extract from *E.coli* that produce USA-CyP was loaded on columns in 0.2 M NaCl, which were washed sequentially with 0.2, 0, 1 and 2.5 M NaCl plus buffer. The columns were eluted with SDS. The columns were H4 peptide (lanes 1–7), H18 peptide (lanes 8–14), H4 peptide in the presence of 25 μM CsA (lanes 15–17) and H4-4M peptide (lanes 18–20). The washes for the last two, which were all blank, are not shown.

E.coli that overproduce USA-CyP was passed over the columns. The columns were washed with salt steps and were eluted with SDS. USA-CyP bound to both the hPrp4 and hPrp18 peptides (Figure 2B, lanes 1–7 and 8–14). Binding was very tight. No USA-CyP eluted from the H4 peptide column with salt (lanes 3–6). USA-CyP could only be eluted from the column with SDS (lane 7). Binding of USA-CyP to the H18 peptide was similar, and SDS was required for elution of USA-CyP from the column. Some USA-CyP eluted from the H18 resin at high salt, probably because poor coupling of the H18 peptide resulted in a low column capacity. USA-CyP did not bind to a blank column (data not shown) or to a column bearing a mutant peptide that differed at four positions from the hPrp4 peptide (H4-4M, lanes 18–20), showing that USA-CyP did not

precipitate on or interact non-specifically with the column. This stable binding is consistent with the observed stability of the complex of USA-CyP with hPrp3 and hPrp4 isolated from HeLa cells (Horowitz *et al*., 1997; Teigelkamp *et al*., 1998). The results show that USA-CyP forms a specific, stable complex with peptides from the homologous regions of hPrp4 and hPrp18.

Geometry of the USA-CyP complexes with hPrp18 and hPrp4

Cyclophilins interact with or act on polypeptides containing proline. The USA-CyP-binding site defined by the homology between hPrp4 and hPrp18 has a completely conserved proline near its center (Figure 1), suggesting that the active site of USA-CyP might bind to the conserved peptide. To test this conjecture, we used CsA, which binds tightly and specifically to the active sites of cyclophilins (Taylor *et al*., 1997). CsA inhibits the prolyl isomerase activity of USA-CyP, and the amino acids in cyclophilins that are important for binding CsA are conserved in USA-CyP (Teigelkamp *et al*., 1998; Reidt *et al*., 2000), implying that CsA binds to the active site of USA-CyP.

We first found that CsA did not interfere with formation of USA-CyP complexes with either hPrp18 or the peptides (Figure 2B, lanes 15–17). This result implied either that binding of USA-CyP to the peptide or to hPrp18 does not involve the active site, or that binding of peptides or hPrp18 to USA-CyP displaces CsA from the complexes. To resolve this question, we used [^{3}H]CsA to determine whether USA-CyP could bind simultaneously to the H4 peptide and CsA. The complex of His-tagged USA-CyP with [^{3}H]CsA was bound to either Ni-NTA–agarose or H4-peptide–agarose. The amounts of bound [^{3}H]CsA were determined by eluting the columns with imidazole (Ni-NTA) or SDS (H4 peptide). Essentially equal amounts of [^{3}H]CsA were eluted from the two columns (Figure 3). This result shows that USA-CyP can bind to both the peptide and CsA at the same time and implies that the active site of USA-CyP is not directly involved in binding the peptide. The quantitative agreement between the numbers further suggests that the geometry of the active site is not significantly altered by binding to the peptide. It is important to note that His-USA-CyP is all monomeric, since dimers or higher aggregates of the protein would complicate the interpretation. Identical results were obtained using His-USA-CyP that was first purified by gel filtration to ensure that multimers were absent (data not shown). In accord with our conclusion, we found that substitution of the conserved proline in the hPrp4 peptide H4 with alanine did not significantly weaken binding of USA-CyP to the peptide (not shown).

In a parallel [^{3}H]CsA binding experiment, the complex of His-hPrp18 and untagged USA-CyP was used (Figure 3). The amount of USA-CyP used was the same as in the above experiments. Nearly equal amounts of [^{3}H]CsA were bound by USA-CyP when it was bound to Ni-NTA, the H4 peptide or hPrp18 (Figure 3), showing that the affinity of USA-CyP for CsA is not altered by formation of the complex with hPrp18 and implying that the strong binding of USA-CyP to hPrp18 does not occur via the active site of USA-CyP. In summary, the CsA binding experiments show that the active site of USA-CyP is exposed in complexes of USA-CyP with the conserved peptides or hPrp18 and suggest that USA-CyP acts on other sites in the spliceosome.

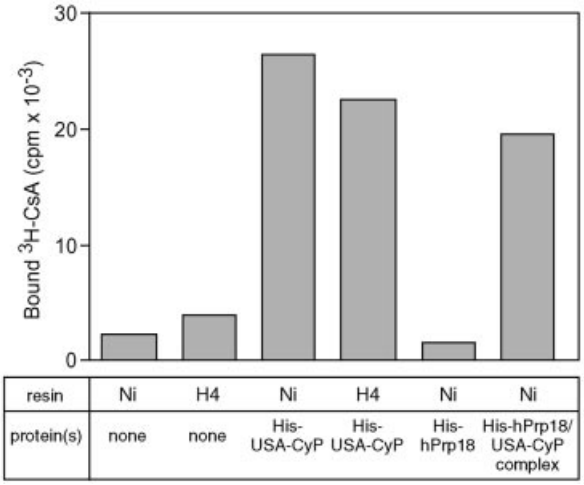

Fig. 3. Binding of [^{3}H]CsA to USA-CyP. [^{3}H]CsA was incubated with the protein(s) indicated at the bottom and then mixed with the resin indicated, where Ni is Ni-NTA–agarose and H4 is H4-peptide–agarose. The amount of [^{3}H]CsA remaining bound to each resin following washing is plotted on the graph.

Complexes of USA-CyP in cell extracts

As the first step toward understanding the function of USA-CyP, we looked for complexes between USA-CyP and hPrp4 or hPrp18 in cell extracts. Antibodies against hPrp3, hPrp4 or hPrp18 (D.S.Horowitz and E.J.Lee, unpublished data) (Horowitz and Krainer, 1997) were used to immunoprecipitate proteins from HeLa cell nuclear extracts, and blots of the immunoprecipitated proteins were probed with anti-USA-CyP (Figure 4A). USA-CyP was co-immunoprecipitated with hPrp3 and hPrp4 (lanes 3 and 4) as part of the previously characterized complex of the three proteins (Horowitz *et al*., 1997; Teigelkamp *et al*., 1998). USA-CyP was also co-precipitated with hPrp18 (lane 6), showing that this complex is present in cell extracts. Anti-USA-CyP does not immunoprecipitate USA-CyP from extracts (lane 5).

The immunoprecipitation experiments were extended to show that the vast majority of the USA-CyP is found in complexes with hPrp3/hPrp4 and hPrp18. Antibodies against hPrp3, hPrp4 or hPrp18 were used independently to deplete extracts of each of these proteins (D.S.Horowitz and E.J.Lee, unpublished data). hPrp3 and hPrp4 were present in roughly equimolar amounts in nuclear extracts and were co-depleted. A western blot of the immunodepleted extracts was probed with anti-USA-CyP (Figure 4B). About half of the USA-CyP was co-precipitated with hPrp3 or hPrp4 (lanes 2 and 3), and about a third of the USA-CyP was co-precipitated with hPrp18 (lane 5). The amount of USA-CyP precipitated by anti-hPrp18 may underestimate the amount of USA-CyP–hPrp18 complex because some USA-CyP may have been displaced by anti-hPrp18, which binds to the USA-CyP-binding site (Horowitz *et al*., 1997). The complexes of USA-CyP with hPrp4 and hPrp18 are distinct; no hPrp4 is precipitated by anti-hPrp18 (Horowitz and Krainer, 1997) nor is

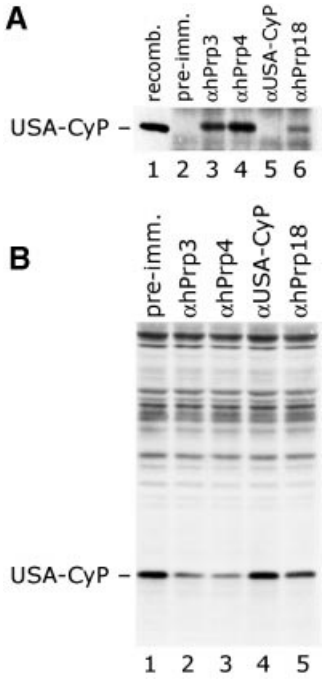

Fig. 4. Complexes of USA-CyP with hPrp3, hPrp4 and hPrp18 in cell extracts. (**A**) Proteins were precipitated from HeLa cell nuclear extract with pre-immune (lane 2), anti-hPrp3 (lane 3), anti-hPrp4 (lane 4), anti-USA-CyP (lane 5) or anti-hPrp18 antibodies (lanes 6) and were analyzed by western blot using anti-USA-CyP. Recombinant USA-CyP was run in lane 1. (**B**) HeLa cell nuclear extract was immunodepleted with pre-immune, anti-hPrp3, anti-hPrp4 or anti-hPrp18 antibodies. The amount of USA-CyP remaining was determined by western blot using anti-USA-CyP.

hPrp18 precipitated by anti-hPrp4 (data not shown). The results show that most of USA-CyP is part of separate complexes with hPrp3/hPrp4 and hPrp18, suggesting that the primary role of USA-CyP is in pre-mRNA splicing.

Cyclosporin A slows splicing in vitro

We used the cyclophilin inhibitor CsA to test the involvement of USA-CyP in pre-mRNA splicing. Splicing of β-globin pre-mRNA by HeLa cell nuclear extract was slowed by the addition of CsA (Figure 5A). The effect of CsA is apparent from comparing the amounts of product mRNA in lanes 1–3 with those in lanes 4–6; products began to form later and formed more slowly. The results are shown in the graph in Figure 5B. At the 40 min time point, 4-fold less mRNA was made in the presence of CsA. As the splicing reaction proceeded, the effect of CsA decreased (Figure 5B); by 180 min, there was little difference among the different reactions (data not shown). Splicing in the presence of CsA 'catches up' with splicing in its absence, consistent with the idea that the effect of CsA is specifically to slow splicing. The inhibition of splicing by CsA appears to result from effects on both steps of splicing: less pre-mRNA was spliced through the first step in the presence of CsA, implying that the first step was inhibited, and there was some accumulation of intermediates from slowing of the second step. The disappearance of pre-mRNA, which is dominated by degradation, was not affected by CsA. The slowing of splicing was reproducibly and reliably seen, and Figure 5 is a representative result. We calculated average effects of CsA from several experiments. The ratios of the amounts of mRNA produced in the absence of CsA to those produced in the presence of CsA were 3.3 ± 0.4 at 40 min, 1.8 ± 0.2 at 65 min and 1.4 ± 0.1 at 90 min. The small standard deviations in these measurements provide strong support for our conclusions. Native gel analysis of the effect of CsA on splicing is provided in the Supplementary data available at *The EMBO Journal* Online.

Inhibition of splicing was measured as a function of concentration of CsA (Figure 5). CsA at 50 and 10 μM significantly slowed splicing, but 2 μM had almost no effect (Figure 5B). Binding constants for CsA binding to cyclophilins can be in the nanomolar range (Taylor *et al*., 1997), and measurement of the inhibition of the prolyl isomerase activity of USA-CyP by CsA suggests tighter binding of CsA to USA-CyP than the micromolar range suggested by our results (Teigelkamp *et al*., 1998). One reason for this apparent discrepancy may be that our reactions are carried out in cell extracts, and the effective concentration of CsA may be lowered by other interactions. More importantly, USA-CyP will form complexes with hPrp4 and hPrp18 in the presence of CsA and will be incorporated into the spliceosome. Thus, CsA must inhibit a reaction between USA-CyP and another component of a single spliceosome (effectively an intramolecular reaction), and a high concentration of CsA may be needed.

USA-CyP functions in the second step of splicing

We sought to determine whether CsA inhibits splicing specifically by blocking the action of USA-CyP. We constructed a mutant hPrp18 lacking the USA-CyP-binding site (called hPrp18ΔCBS for hPrp18 deleted for the cyclophilin-binding site); we hypothesized that the second step of splicing using hPrp18ΔCBS would be insensitive to CsA since USA-CyP would not interact with hPrp18ΔCBS. hPrp18ΔCBS was designed based on the X-ray crystal structure of the yeast Prp18 fragment Prp18Δ79, which lacks the 79 N-terminal amino acids of Prp18 and is fully active in yeast splicing *in vitro* (Jiang *et al*., 2000). hPrp18ΔCBS therefore contains the evolutionarily conserved C-terminal portion of hPrp18 and lacks the N-terminal 155 amino acids of hPrp18, including the 31 amino acid USA-CyP-binding site.

We used hPrp18ΔCBS to test whether USA-CyP played a role in the second step of splicing. Splicing by extracts immunodepleted of hPrp18 is blocked after the first step of splicing, and splicing activity can be restored by the addition of hPrp18 protein (Horowitz and Krainer, 1997). Time courses of splicing were carried out in hPrp18-depleted extracts with and without 50 μM CsA (Figure 5C, lanes 1–6), and in hPrp18-depleted extracts that had been reconstituted with hPrp18 or hPrp18ΔCBS, both with and without CsA (Figure 5C, lanes 7–18). The kinetics of splicing in the reconstituted extracts were slightly different from those in untreated extracts. In the absence of hPrp18, the first step could be assayed specifically, and only a small effect of CsA was observed under these conditions (comparing lanes 1–3 with 4–6 in Figure 5C). The reduced effect of CsA on the first step, which we attribute to the overall slowing of the first step caused non-specifically by the depletion procedure, allowed us to focus on the effect of CsA on the second step.

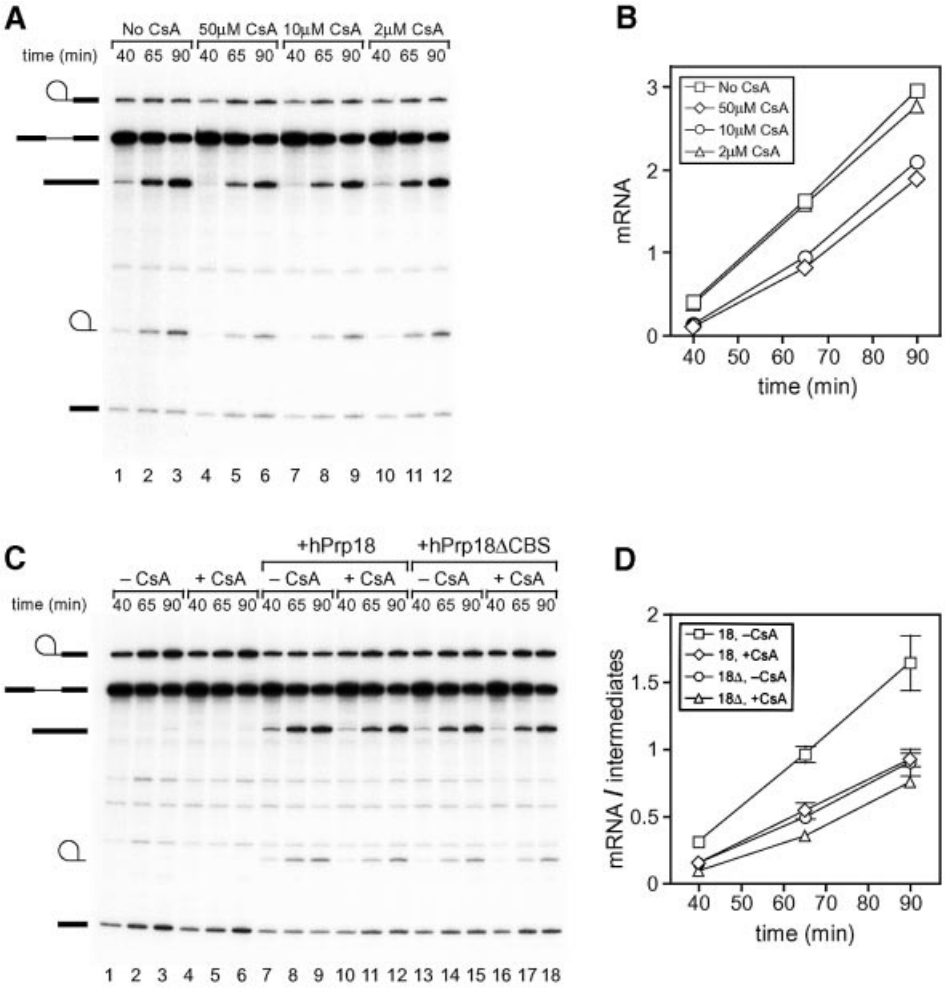

Fig. 5. Inhibition of splicing *in vitro* by CsA. (**A**) Splicing of β-globin pre-mRNA was assayed in the presence of 0, 50, 10 or 2 μM CsA for the times shown at the top of each lane. The products were resolved in a 9% polyacrylamide–urea gel. (**B**) Rate of formation of mRNA (in arbitrary units) at different concentrations of CsA. The amount of mRNA from each lane of (A) is shown. Shown are no CsA (squares), 2 μM CsA (triangles), 10 μM CsA (circles) and 50 μM CsA (diamonds). (**C**) Splicing was assayed in extracts immunodepleted of hPrp18 following addition of 50 μM CsA, hPrp18 or hPrp18ΔCBS, as indicated at the top of the panel. The reaction times are shown at the top of each lane. (**D**) Molar ratio of mRNA to lariat intermediate versus time averaged from two independent experiments. This ratio is a measure of the efficiency of the second step. Shown are +hPrp18, –CsA (squares), +hPrp18, +CsA (diamonds), +hPrp18ΔCBS, –CsA (circles) and +hPrp18ΔCBS, +CsA (triangles).

In extracts reconstituted with hPrp18, the effect of CsA on the second step was apparent from comparing the amounts of intermediates and products in lanes 7–9 with those in lanes 10–12. As a measure of the rate of the second step, we plotted the molar ratio of mRNA to intermediates as a function of time (Figure 5D). The results in Figure 5C were obtained reproducibly, and average mRNA:intermediates ratios with experimental errors are shown in Figure 5D. Because CsA has only a small effect on the first step under these conditions, this ratio is a reasonable measure of the efficiency of the second step. The graph and the gel show that CsA inhibited the second step when wild-type hPrp18 was used. In contrast, in extracts that had been reconstituted with hPrp18ΔCBS, CsA did not affect the second step of splicing (comparing lanes 13–15 with lanes 16–18), showing that deletion of the USA-CyP-binding site from hPrp18 abolished the inhibitory effect of CsA on the second step. The absence of a CsA effect on the second step when hPrp18ΔCBS was used strongly suggests that CsA inhibits the second step by blocking the action of USA-CyP, since USA-CyP is not expected to play any role in the second step when hPrp18ΔCBS is used. In addition, the rate of the second step was essentially equal using wild-type hPrp18 with CsA or hPrp18ΔCBS without CsA, i.e. preventing USA-CyP from acting during the second step by deleting its binding site is functionally equivalent to adding CsA to inhibit USA-CyP. The results imply that the inhibition of the second step of splicing by CsA is caused by CsA inhibiting USA-CyP bound to hPrp18.

Cyclosporin A affects pre-mRNA splicing in vivo

To test the effect of CsA on pre-mRNA splicing *in vivo*, we applied a previously characterized transcript release assay using fluorescence *in situ* hybridization (FISH) (Custodio *et al.*, 1999). The assay is based on the requirement for completion of pre-mRNA splicing prior to release of a transcript from its site of transcription. Upon treatment of cells with transcription inhibitors, fully

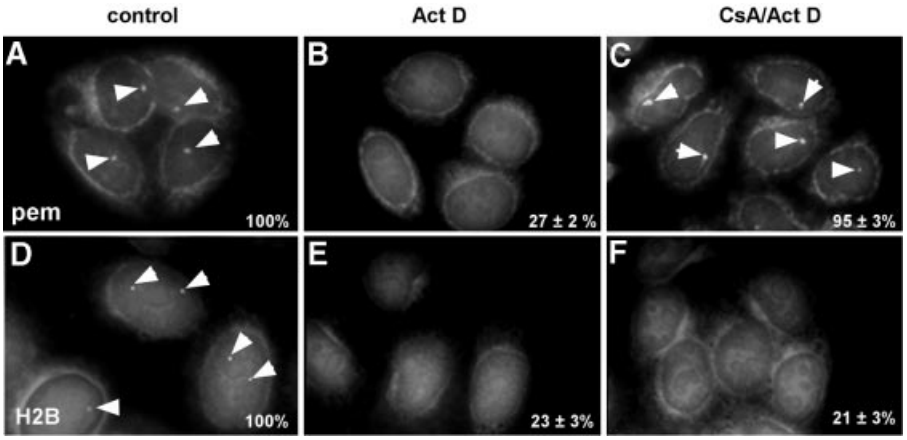

Fig. 6. CsA prevents release of RNA from the sites of transcription. *pem* or histone H2B RNAs were visualized in HeLa cells by FISH following treatment with (**A** and **D**) no drug, (**B** and **E**) actinomycin D (Act D) or (**C** and **F**) CsA followed by Act D. Sites of transcription are indicated by arrowheads. The percentage of cells positive for the *pem* or H2B signal, averaged from at least three experiments, is shown in each panel. Values were normalized to the percentage of positive cells in the control sample (40–60% for *pem*, and 20–30% for H2B) and are shown with their SEMs.

processed transcripts are rapidly lost from their sites of transcription, while unspliced transcripts are retained. The fate of a particular RNA can be visualized by FISH. As expected, in a HeLa cell line stably expressing a single copy of the integrated rat *pem* gene, the *pem* RNA localized in one strongly labeled spot in the cell nucleus representing the site of *pem* transcription (Figure 6A) (Misteli and Spector, 1999). Upon treatment of pemHeLa cells with actinomycin D for 30 min, the population of cells positive for the FISH signal decreased significantly (Figure 6B). The loss of RNA from the transcription site upon actinomycin D treatment was prevented by pre-treatment of cells with CsA for 3 h (Figure 6C). Under these conditions, the fluorescent spot persisted and the number of cells showing a FISH signal was similar to the control population (Figure 6A). Identical results were obtained with 25 or 50 μM CsA (data not shown). The effect of CsA depended on the presence of an intron in the pre-mRNA, since the RNA of the histone H2B gene, which lacks introns, was not retained at the site of transcription upon CsA treatment (Figure 6D–F). These observations show that CsA interferes with the release of newly synthesized intron-containing transcripts from their site of transcription.

To demonstrate that the accumulation of RNA was a consequence of inhibition of pre-mRNA splicing by CsA, pemHeLa cells were treated with CsA, and FISH was performed using specific oligonucleotide probes to detect unspliced and spliced *pem* RNA (Misteli and Spector, 1999). To detect unspliced RNA, we used an oligonucleotide against a sequence in intron 4 of *pem*, and to detect spliced RNA we used an oligonucleotide complementary to the 3′ end of exon 4 and the 5′ end of exon 5. A control oligonucleotide containing the two exon sequences in reverse order gave no signal (data not shown). The intron probe gave a specific signal at the site of transcription regardless of whether cells had been treated with CsA, indicating the presence of unspliced RNA at the site of transcription (Figure 7A, panels a and c). In contrast, no spliced RNA was detected in CsA-treated cells (Figure 7A, panel d). Identical results were observed with the splice junction probes in cells treated with CsA followed by actinomycin D (data not shown). The accumulation of unspliced RNA shows directly that CsA prevents splicing of the *pem* pre-mRNA *in vivo*.

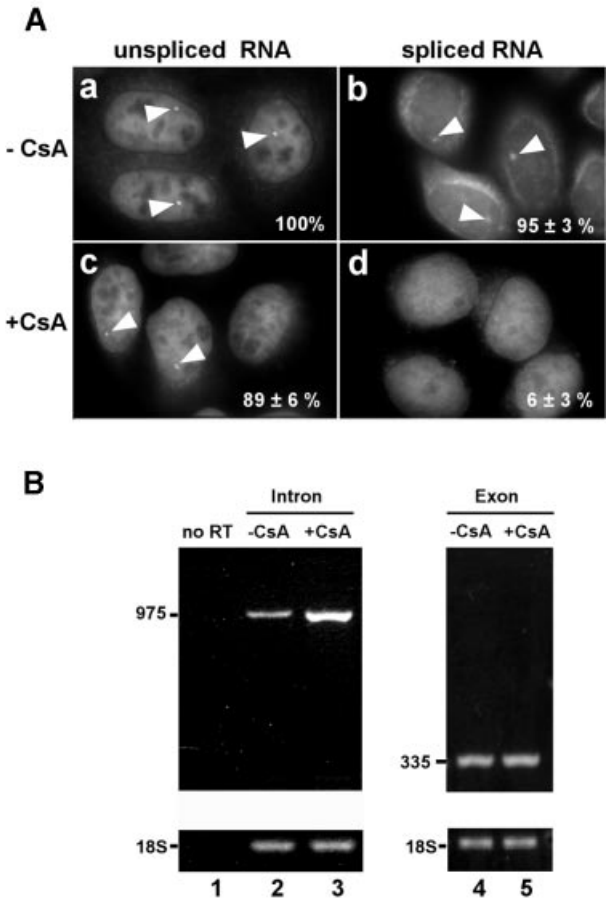

Fig. 7. Splicing of *pem* pre-mRNA is inhibited by CsA. (**A**) *pem* RNA was visualized by FISH with an intron 4 probe which is specific for unspliced RNA, or an exon 4/5 probe which is specific for spliced RNA, following treatment with (a and b) no drug or (c and d) CsA. Values are averages of at least three experiments, and errors represent SEM. (**B**) *pem* RNA from pemHeLa cells was analyzed by RT–PCR using primers in the introns flanking exon 5 to amplify unspliced pre-mRNA (lanes 2 and 3) and primers within exon 4 to amplify total *pem* RNA (lanes 4 and 5). 18S RNA was amplified separately as a standard.

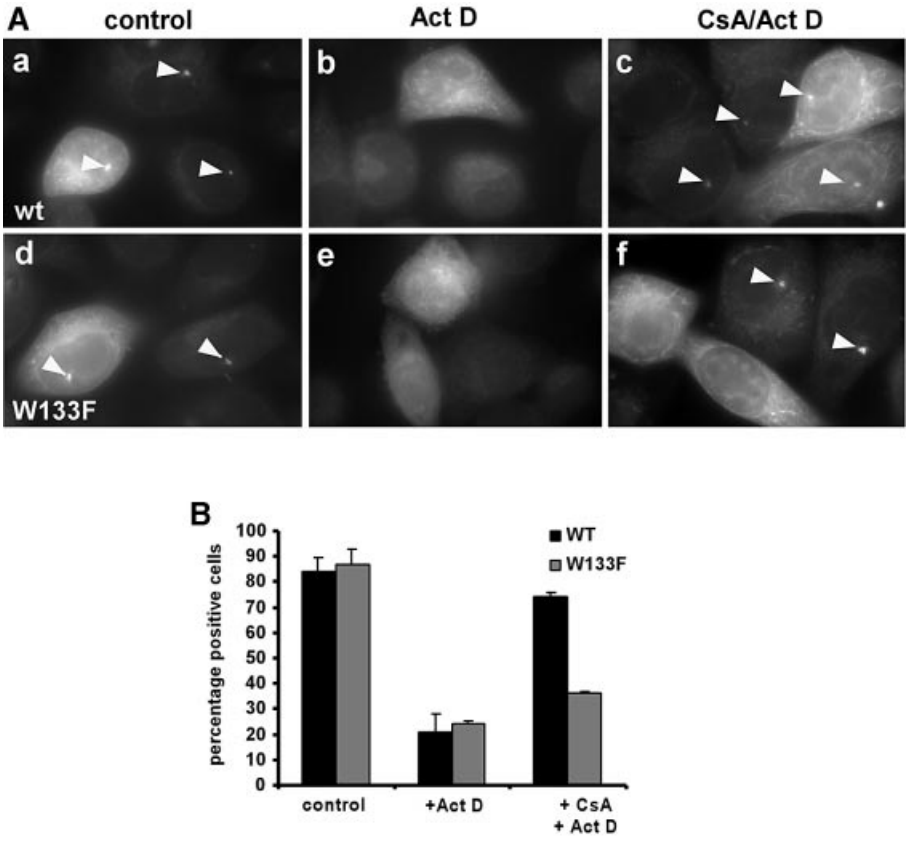

Fig. 8. CsA inhibits USA-CyP *in vivo*. (**A**) pemHeLa cells were co-transfected with either wild-type USA-CyP (a–c) or USA-CyP-W133F (d–f) together with EYFP-Mito, which served as a transfection marker. *pem* RNA was visualized by FISH following treatment with (a and d) no drug, (b and e) actinomycin D (Act D) or (c and f) CsA followed by Act D. Transfected cells can be identified by their cytoplasmic signal, and sites of transcription are indicated by arrowheads. (**B**) Percentage of transfected cells in which the *pem* signal was seen. Values are averages of at least three experiments, and error bars represent the SEM.

The inhibition of splicing of *pem* RNA *in vivo* was confirmed by RT–PCR (Figure 7B). To detect unspliced RNA, we used primers in the introns on either side of exon 5 (lanes 2 and 3). A PCR product can only be generated from unspliced RNA. In cells treated with CsA, an ~2.5-fold increase in the RT–PCR signal was detected compared with untreated cells. This increase was not due to an overall increase in *pem* transcription since primers within exon 5 gave identical levels of PCR product (lanes 4 and 5). Taken together, the FISH and RT–PCR analyses demonstrate that CsA inhibits pre-mRNA splicing *in vivo*.

USA-CyP functions in pre-mRNA splicing in vivo

To determine whether the inhibition of splicing by CsA was caused specifically by inhibition of the action of USA-CyP, we designed a CsA-resistant mutant of USA-CyP. The tryptophan residue corresponding to Trp133 of USA-CyP is critical for binding of CsA by cyclophilins (Liu *et al*., 1991; Taylor *et al*., 1997). Mutation of this tryptophan to phenylalanine in several cyclophilins significantly reduced their affinities for CsA, while only modestly affecting their enzymatic and biological activities (Liu *et al*., 1991; Dorfman *et al*., 1997). Our binding results imply that residues important for CsA binding, such as Trp133, are not important for the formation of stable complexes with hPrp4 or hPrp18. Furthermore, this tryptophan is only partly conserved among cyclophilins (Galat and Rivière, 1998), and, more importantly, among five homologs of USA-CyP three have phenylalanine (Horowitz *et al*., 1997; Teigelkamp *et al*., 1998; unpublished data).

Wild-type and USA-CyP-W133F were transiently expressed in pemHeLa cells. A plasmid that directs the production of EYFP fused to a mitochondrial targeting signal was co-transfected into the cells as a marker for transfected cells, which are evident from their green fluorescence signal in the cytoplasm (Figure 8A). Splicing of the *pem* pre-mRNA was assayed using the transcript release assay. Cells transfected with wild-type USA-CyP (wt cells; Figure 8A, panels a–c) behaved similarly to untransfected cells (compare Figure 6A–C with Figure 8A, panels a–c, or compare transfected and untransfected cells in Figure 8A, a–c). In cells transfected with USA-CyP-W133F (W133F cells), *pem* pre-mRNA was detected at its sites of transcription (Figure 8A, panel d), and the *pem* RNA disappeared after treatment with actinomycin D (Figure 8A, panel e), just as in wild-type and untransfected cells. However, in contrast to wild-type cells (Figure 8A, panel c), *pem* RNA did not accumulate in W133F cells treated sequentially with CsA and actinomycin D (panel f). The percentages of transfected cells positive for FISH signals are shown in Figure 8B. As an internal control, in untransfected cells, *pem* pre-mRNA did accumulate (panel f). These results imply that USA-CyP-W133F is

working properly and is resistant to CsA. They suggest that the inhibition of splicing by CsA *in vivo* is caused specifically by blocking the actions of USA-CyP.

Discussion

The functions of cyclophilins and other immunophilins have proven difficult to identify (Marks, 1996; Hunter, 1998). Compounding the mystery surrounding the cellular roles of the immunophilins are uncertainties about how they act and whether they are prolyl isomerases or chaperones (Schreiber and Crabtree, 1992; Luban, 1996; Ivery, 2000; Schiene and Fischer, 2000). We have found that the cyclophilin USA-CyP functions in pre-mRNA splicing. Both *in vitro* and *in vivo* results support this conclusion and further suggest that the primary cellular role of USA-CyP is in splicing. Our results provide important mechanistic insights into the function of USA-CyP. USA-CyP is bound tightly to two different proteins within the spliceosome, and from these two sites USA-CyP can act on its targets in the spliceosome. USA-CyP probably mediates conformational changes of proteins within the spliceosome. The combination of functional and mechanistic data that we have obtained makes USA-CyP one of the best understood cyclophilins.

Complexes of USA-CyP with hPrp4 and hPrp18

We characterized two stable complexes of USA-CyP, one with hPrp4 and the other with hPrp18. The complex of USA-CyP and hPrp4 had been identified previously (Horowitz *et al*., 1997; Teigelkamp *et al*., 1998), but finding the complex of USA-CyP with hPrp18 was unexpected. hPrp4 and hPrp18 have a 31 amino acid region of homology that is evolutionarily conserved (Figure 1). The homologous regions are high affinity binding sites for USA-CyP in each protein. Outside of their USA-CyP-binding sites, both the sequences and structures of hPrp4 and hPrp18 are unrelated (Ayadi *et al*., 1998; Jiang *et al*., 2000). hPrp4 and hPrp18 act in different steps of splicing and appear to have unrelated functions. The very stable interaction of USA-CyP with hPrp4 or hPrp18 suggests that USA-CyP is not released from the proteins and remains bound to them throughout splicing. The active site of USA-CyP is exposed in these complexes, meaning that binding to hPrp4 or hPrp18 occurs through another part of the cyclophilin and implying that the sites on which USA-CyP acts are elsewhere in the spliceosome.

Most of the USA-CyP in nuclear extracts is present in complexes with hPrp4 and hPrp18, suggesting that the principal or only function of USA-CyP is in these complexes. No sequences homologous to the USA-CyP-binding sites in hPrp4 and hPrp18 were found elsewhere in the available human sequences, consistent with the idea that these two proteins are the only sites of USA-CyP binding. Our observation that USA-CyP appears to be a 'splicing-specific' cyclophilin supports the idea that each cyclophilin has a limited number of specific functions, although highly expressed cyclophilins such as CyP A may have more diverse roles (Marks, 1996).

USA-CyP functions during pre-mRNA splicing

We used the cyclophilin inhibitor CsA together with our knowledge of the USA-CyP-binding sites to show that USA-CyP functions in pre-mRNA splicing both *in vitro* and *in vivo*. CsA slows splicing ~2-fold *in vitro*. Both steps appear to be slowed to give rise to this effect, as might be expected if USA-CyP is the target. To show directly that USA-CyP participates in splicing, we focused on understanding its role in the second step, involving hPrp18, for which we have superior tools (Horowitz and Krainer, 1997). A mutant form of hPrp18 that lacks the USA-CyP-binding site (hPrp18ΔCBS) retains partial activity but is insensitive to CsA. The efficiency of the second step was the same using hPrp18 with CsA or hPrp18ΔCBS with or without CsA. These results show that USA-CyP plays a role in the second step of splicing and that formation of the complex between USA-CyP and hPrp18 is essential for USA-CyP action. We do not have the tools to carry out a parallel investigation of the function of USA-CyP bound to hPrp4, but we think that CsA inhibition of the first step of splicing is caused by inhibition of USA-CyP bound to the complex of hPrp4 and hPrp3. Our results paint a consistent picture in which USA-CyP accelerates both steps of splicing from within its two complexes.

In parallel experiments, we characterized the role of USA-CyP *in vivo*. We used both the transcript release assay and RT–PCR to show that CsA inhibited splicing in cells. This inhibition results from blocking the function of USA-CyP. When the mutant USA-CyP-W133F, which is expected to be functional and CsA resistant, was overexpressed in HeLa cells, splicing was not inhibited by CsA, implying that USA-CyP was the target of CsA. These results argue strongly that USA-CyP functions in pre-mRNA splicing *in vivo*.

Roles in splicing have been suggested for the cyclophilins CARS-CyP (also called SRcyp and matrin CYP) and inferentially for the related NK-TR protein (Nestel *et al*., 1996), as well as for the *Caenorhabditis elegans* Cyp-13 protein (Zorio and Blumenthal, 1999). While not excluding a role for these cyclophilins in splicing, our results point directly to a role for USA-CyP in splicing.

It is easy to understand why a cyclophilin could be needed for splicing: the spliceosome undergoes many structural rearrangements during splicing, and a cyclophilin could help to manage protein conformations during these changes. In splicing, a prolyl isomerase could trigger or facilitate changes in the geometry of the spliceosome; a chaperone could be needed to prevent inappropriate interactions of proteins.

A variety of roles can be suggested for USA-CyP based on its presence in the complex with hPrp3 and hPrp4. Some cyclophilins and immunophilins are involved in the folding and transport of proteins (Baker *et al*., 1994; Schiene and Fischer, 2000). Our results show that USA-CyP plays a direct role in splicing. hPrp4 and hPrp18 bring USA-CyP into the spliceosome to act at two different times in splicing. USA-CyP could facilitate the entry of hPrp18 into the spliceosome by altering the conformation of a spliceosomal protein, could affect conformations of the spliceosome during the second step or could assist in the disassembly of the spliceosome. hPrp4 enters and exits the spliceosome with the U4 snRNP (Ayadi *et al*., 1997),

and a similar set of roles for USA-CyP is possible in this case.

Despite the conservation of USA-CyP and its binding sites over large evolutionary distances, USA-CyP appears to have a modest effect on pre-mRNA splicing *in vitro*. *In vitro* splicing takes tens of minutes, while spontaneous isomerization of prolyl peptide bonds occurs in seconds to minutes (Stein, 1993). Thus, if USA-CyP acts by isomerizing a prolyl bond, then USA-CyP's role in splicing *in vitro* may be masked by the relatively rapid spontaneous isomerization of the bond. In cells, on the other hand, splicing occurs much more rapidly, and the effect of USA-CyP may be much larger. Our *in vivo* data show that CsA has a clear inhibitory effect on pre-mRNA splicing, although the transcript release assay does not allow us to estimate the efficiency of splicing *in vivo*. The subtle effect of USA-CyP observed may help to explain why so few functions for cyclophilins have been identified.

Mechanistic comparisons with other cyclophilins

USA-CyP forms a stable complex with hPrp4 or hPrp18 in which its active site is exposed. Closely related (70–75% identical) homologs of USA-CyP are found in *C.elegans* and *Drosophila melanogaster*. Two prominent conserved regions in the USA-CyPs that are different from other cyclophilins are the C-terminal 25 amino acids and a five amino acid insertion at position 51 (Horowitz *et al*., 1997; Galat and Rivière, 1998; Teigelkamp *et al*., 1998). These regions may play a role in the interaction of USA-CyP with hPrp4 and hPrp18. Reidt *et al*. (2000) have solved the structure of USA-CyP and suggest that the inserted amino acids form part of a hydrophobic cleft that could interact with other proteins. Small cyclophilins like USA-CyP are often hypothesized to interact with other proteins principally or exclusively via their active sites, as is seen in the crystal structures of CyP A with the human immunodeficiency virus (HIV) capsid protein, and of the immunophilin FKBP12 with transforming growth factor-β (Gamble *et al*., 1996; Zhao *et al*., 1997; Huse *et al*., 1999). At least in the case of HIV, there is little specificity in the binding, and any cyclophilin will bind to the HIV capsid protein (Braaten and Luban, 2001). Our results suggest a model in which USA-CyP binding occurs primarily away from the active site, setting up the cyclophilin to act on a specific target. Whether other small cyclophilins might operate similarly is not known. Some larger cyclophilins have been found within stable complexes in which their active sites are exposed (Leverson and Ness, 1998).

The anchoring of USA-CyP to hPrp4 and hPrp18 sets up its actions elsewhere in the spliceosome. USA-CyP need not have high affinities for its target sites; its sites of action may be governed by its proximity to them in the spliceosome. Our results do not define the sites of action of USA-CyP, and there are many potential targets. USA-CyP has two functions in splicing, one associated with each of its complexes. One interesting possibility is that the reason that USA-CyP is bound to both hPrp3/hPrp4 and hPrp18 is that these are its targets of action. The most conserved region of the Prp18s forms a flexible loop, which could be a site of USA-CyP action (Jiang *et al*., 2000). hPrp4 appears to be an unlikely target, because it is a WD-repeat protein, probably with a rigid structure; however, hPrp3, which is tightly bound to hPrp4, could be a target.

Table I. Calculated and measured molecular weights of proteins and protein complexes

Protein	Calculated mol. wt	Measured mol. wt
His-hPrp18	42 600	52 000
USA-CyP	19 200	16 000
His-USA-CyP	22 000	19 000
His-hPrp18 + His-USA-CyP	61 800	72 000

A limited number of cyclophilin actions have been characterized (Luban, 1996; Marks, 1996; Hunter, 1998; Schiene and Fischer, 2000). The proposed mechanism of action of CyP A in HIV infection contrasts with our results for USA-CyP. CyP A is required for infectivity of the HIV virion, and forms a stable complex with the HIV capsid protein through its active site, leading to the idea that CyP A acts as a chaperone (Luban, 1996). CyP A may facilitate disassembly of the viral capsid (Gamble *et al*., 1996; Zhao *et al*., 1997) or it may act as an adaptor linking the virus to the cell membrane (Saphire *et al*., 1999). Our results for USA-CyP are quite different. Its stable complex with hPrp4 and hPrp18 does not involve its active site. It is unclear whether USA-CyP and CyP A/HIV represent superficially different examples of a common mechanism or are in fact different ways that cyclophilins can act. The mechanism we propose, in which USA-CyP is ferried into the spliceosome by hPrp4 and again by hPrp18, can be reconciled with USA-CyP acting either as a prolyl isomerase or as a chaperone. The mechanism of action of USA-CyP is similar to that proposed for CyP-40 and c-Myb, in which the TPR domain of CyP-40 interacts stably with c-Myb, and the cyclophilin acts within this complex on c-Myb (Leverson and Ness, 1998).

We have identified a function for the cyclophilin USA-CyP. As part of two distinct complexes with other splicing factors, USA-CyP acts in both steps of pre-mRNA splicing. The mechanism of action that we propose for USA-CyP, in which its stable binding at one site sets up its actions at another, may be applicable to other cyclophilins. USA-CyP is likely to mediate conformational changes of proteins in the spliceosome. Whether other cyclophilins play related roles in other complex processes remains to be determined.

Materials and methods

Production of recombinant proteins

Wild-type and His-tagged USA-CyP were produced in *E.coli* using pET9a and pET19b (Novagen). Proteins were produced at 18°C in the presence of 2% ethanol (Higman *et al*., 1992) to avoid the insolubility problems previously reported (Teigelkamp *et al*., 1998). Cells were lysed with lysozyme/Brij-58, nucleic acid was removed with polyethyleneimine, and proteins were precipitated with $(NH_4)_2SO_4$. His-USA-CyP was purified further on Ni-NTA–agarose (Qiagen). The sizes of both forms of USA-CyP (Table I) were measured using a 30 cm Superdex-75 column (Pharmacia) in 25 mM Tris pH 7.5, 200 mM NaCl, 10% glycerol, 1 mM β-mercaptoethanol (buffer Q200) using Sigma MW-GF-200 standards plus myoglobin and ovalbumin. Antibodies were raised in rabbits against His-USA-CyP that had been purified on Ni-NTA–agarose under denaturing conditions.

His-tagged hPrp18ΔCBS was produced in *E.coli* using pET19b. hPrp18ΔCBS was soluble and was purified as described above for

His-USA-CyP. His-hPrp18 was prepared as described (Horowitz and Krainer, 1997).

Complexes of USA-CyP with hPrp18 and peptides

The complex of His-hPrp18 and USA-CyP was formed by incubating the proteins (or extracts) in buffer Q250 at 30°C for 30 min. The complex was purified in this buffer using Ni-NTA–agarose and dialyzed for use in CsA-binding experiments. The sizes of His-hPrp18 and of its complexes with His-USA-CyP were determined by gel filtration as described above (Table I) except that the concentration of NaCl was 1 M to prevent His-hPrp18 from interacting with the column.

The peptide H4 CEVKASLRALGEPITLFGEGPAERRERLRNI has the sequence shown for hPrp4 in Figure 1 except that a cysteine was added to the N-terminus for coupling, serine was substituted for an internal cysteine to avoid interference with coupling, and the C-terminal leucine, which is not conserved in hPrp18, was omitted. Analogous changes were made for the hPrp18-derived peptide H18 CEVIRRLRERGEPIRLFGETDYDAFQRLRKI except that no internal residues were changed. There are four (underlined) changes from H4 in the H4-4M mutant peptide CKVKASLDALGEPITLFGKGPAERREDLRNI. Peptides were coupled to SulfoLink Coupling Gel (Pierce). Peptide columns were run in buffer Q200. All peptides were verified by complete sequencing.

CsA-binding assays

[^{3}H]CsA (2 μCi; Amersham) was incubated at 30°C for 45 min with 2 μg of His-USA-CyP, 4 μg of His-hPrp18 or 6 μg of His-hPrp18 complex with USA-CyP in buffer Q200 plus 0.02% NP-40 (Calbiochem). The mixtures were subsequently incubated for 45 min with H4 peptide–agarose or Ni-NTA–agarose. The resins were washed four times with Q200 plus NP-40, and eluted with SDS or imidazole for counting.

In vitro splicing

Preparation of HeLa cell nuclear extracts and splicing of β-globin were carried out as described previously (Mayeda and Krainer, 1999). We used 40 mM KCl for splicing because this KCl concentration gave larger inhibitory effects with CsA and did not significantly affect splicing efficiency. CsA (Calbiochem) in dimethylsulfoxide (DMSO) was added to nuclear extracts, which were incubated at 4°C for 1 h prior to the initiation of splicing. The concentration of DMSO was 1% in all splicing reactions.

hPrp18-depleted extracts were prepared as described (Horowitz and Krainer, 1997). A 1.5 ng aliquot of hPrp18 or 5 ng of hPrp18ΔCBS per μl nuclear extract was added to reconstitute splicing. Titration or hPrp18 and hPrp18ΔCBS used for reconstitution showed that each protein had a broad plateau of maximal activity. The amounts used were from the plateau regions of the curves. CsA was added as above prior to the addition of hPrp18 or hPrp18ΔCBS. RNAs were quantitated with a Molecular Dynamics PhosphorImager. Statistical evaluation of mRNAs was based on 3–6 independent experiments.

Transcript release assay and transfection

The transcript release assay was carried out essentially as described (Custodio *et al*., 1999). Cells were incubated with medium containing either 25 or 50 μM CsA (from a 10 mM stock in ethanol or DMSO) for 3 h or with an equivalent volume of solvent. Where indicated, cells were treated for 30 min with 25 μg/ml actinomycin D before fixation. RNA was detected essentially as described (Misteli and Spector, 1999). Full-length, biotinylated probes or oligonucleotide probes were incubated with fixed cells in 2× SSC, 10% dextran sulfate, 1 mg/ml yeast tRNA and 50% formamide. Following washing, probes were detected with avidin-fluorescein isothiocyanate (FITC). Images were acquired on a Nikon E800 microscope equipped with a MicroMax cooled CCD camera using Metamorph 4.0 (Universal Imaging).

The W133F mutant of USA-CyP was made using the QuikChange Kit (Stratagene) and was cloned into pcDNA3.1+ (Invitrogen) for expression. pemHeLa cells were transfected with 2 μg of pDH133 (USA-CyP-W133F) or pDH136 (wild type) and 1 μg of EYFP-Mito (Clontech) by electroporation, and were grown for 18 h before use. Pilot experiments using Xpress-tagged USA-CyP (Invitrogen) and EYFP-Mito established that this ratio of plasmids gave protein expression from both plasmids in the same cells.

RT–PCR

RNA isolated from pemHeLa cells using RNAwiz (Ambion) was reverse-transcribed with MuLV reverse transcriptase (Perkin-Elmer) using random decamer primers (Ambion). Thirty cycles of PCR using Amplitaq Gold Polymerase (Perkin-Elmer) were used for detection of total RNA, and 36 cycles for unspliced RNA. For detection of total *pem* RNA, the primers pemE4f: 5′-CAGAGGCTTTCTTTCAGGCTGCAGA and pemE4r 5′-GCCACTGGAGGAACCACTGCTTAGC within exon 4 were used, yielding a 335 bp product. For detection of unspliced RNA, the primers pemI4f 5′-TTAAACCTCAAAGTGGATGTTACGA and pemI5r 5′-tcgtcacctccagagtgctggcact in the introns flanking exon 5 were used, yielding a 975 bp product. Alternate 18S primers (Ambion) were used for internal standardization.

Supplementary data

Supplementary data for this paper are available at *The EMBO Journal* Online.

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