Available online at www.sciencedirect.com
SCIENCE DIRECT®
Journal of Chromatography B, 786 (2003) 81–91
JOURNAL OF CHROMATOGRAPHY B
www.elsevier.com/locate/chromb

Identification and characterisation of *Schizosaccharomyces pombe* cyclophilin 3, a cyclosporin A insensitive orthologue of human USA-CyP

Trevor J. Pemberton[a,*], Stuart L. Rulten[b], John E. Kay[a]

[a]*The School of Biological Sciences, University of Sussex, Falmer, Brighton, East Sussex, BN1 9QG, UK*
[b]*The Trafford Centre for Medical Research, University of Sussex, Falmer, Brighton, East Sussex, BN1 9QG, UK*

Abstract

We have identified nine cyclophilins encoded in the genome of the fission yeast *Schizosaccharomyces pombe* (Sp). Cyclophilin 3 is an orthologue of hUSA-CyP, which is associated with Prp4/Prp3 in the [U4/U6·U5] snRNP complex and Prp18, both of which are components of the pre-mRNA splicing machinery. PPIase assays have shown SpCyp3 and hUSA-CyP to have comparable activity and substrate specificity, but SpCyp3 has a reduced sensitivity to CsA correlating with a difference in the catalytic site. Prp3, Prp4 and Prp18 proteins exist in *S. pombe* and nuclear localisation of SpCyp3 has been shown, indicating conservation of function between hUSA-CyP and SpCyp3.

Keywords: *Schizosaccharomyces pombe*; Cyclophilin; Peptidyl-prolyl *cis*/*trans* isomerase (PPIase)

1. Introduction

Cyclophilins (cyclosporin A binding proteins; Cyps) are one of three known families that make up the peptidyl-prolyl *cis*/*trans* isomerase (PPIase) class of proteins [1]. These proteins catalyse the *cis*/*trans* isomerisation of a peptide bond preceding a proline residue and are widely distributed in eukaryotes, prokaryotes and archaea [2,3]. Cyclophilins are identified by their sensitivity to cyclosporin A (CsA) [4,5], an immunosuppressant drug of fungal origin used in transplant therapy, and inter- and intra-species sequence conservation between members of this family [6,7]. More recently cyclophilins have been linked to HIV infection [8–11], cancer [12], heat-shock recovery [13] in cells and neuropathies [14].

Cyclosporin A is an important immunosuppressive drug and its activity requires an interaction with a cyclophilin. Immunosuppression is caused by a cyclophilin–CsA complex inhibiting the Ca^{2+}-dependent protein phosphatase calcineurin [4,15,16]. The complex binds to the alpha sub-unit of calcineurin causing the inhibition of its activity. Proliferation of activated T cells is dependent upon the expression of the cytokine interleukin-2 [17,18]. Interleukin-2 transcription is activated by the transcription factor NF-AT, whose formation is due to the localisation of the cytoplasmic component to the nucleus through a calcineurin-initiated pathway. Blocking this pathway leads to the inhibition of

*Corresponding author. Tel.: +44-1273-678923; fax: +44-1273-678433.
E-mail address: t.j.pemberton@sussex.ac.uk (T.J. Pemberton).

1570-0232/02/$ – see front matter
doi:10.1016/S1570-0232(02)00738-9

T-cell proliferation and therefore immunosuppression.

The genome of the fission yeast *Schizosaccharomyces pombe* has recently been completed by the sequencing consortium led by the Sanger Centre [19]. Some pathways within the *S. pombe* cell have been found to show homology to similar pathways in the human cell. This has led to it becoming an important model organism for research into human disease. We have identified that nine cyclophilins of varying complexity are encoded in the *S. pombe* genome, with some having close human homologues.

We report below that *S. pombe* cyclophilin 3 is an orthologue [20] of human USA-CyP (also called cyclophilin 20), a 20×10^3 rel. mol. mass protein that is a member of the pre-mRNA splicing complex. hUSA-CyP has been shown to associate with the hPrp4/hPrp3 complex that forms an integral part of the U4/U6 small nuclear ribonucleoprotein (snRNP) complex [21,22] and more recently with hPrp18 [23]. A 31 amino acid homologous region found in both Prp4 and Prp18 has been shown to be the binding site for hUSA-CyP [23]. Treatment with cyclosporin A has been shown to slow both stages of pre-mRNA splicing [23]. Mutagenesis of hUSA-CyP to produce a CsA-resistant variant made the splicing process resistant to CsA treatment [23], showing that the activity of hUSA-CyP is essential for the normal functioning of the pre-mRNA splicing complex.

We report here the identification of the *S. pombe* cyclophilin repertoire and the cloning, expression and characterisation of *S. pombe* cyclophilin 3 in comparison with human USA-CyP.

2. Experimental and results

2.1. Identification of the S. pombe cyclophilins

The cyclophilins of *S. pombe* were identified using the *S. pombe* BLAST [24] server at the Sanger Centre. The protein sequence of human cyclophilin A (Accession # P05092) was used as the query sequence on a BLASTP search of the predicted *S. pombe* proteins database that identified nine putative cyclophilin sequences. A TBLASTN search of the genomic DNA database was also carried out using the human cyclophilin A protein sequence as a probe to ensure no cyclophilins had been missed during the sequence annotation by the Sanger Centre. Only the nine sequences identified by the BLASTP search were found. Each putative cyclophilin sequence in turn was then used as the query sequence in a BLASTP search of the Sanger database. As they all identified the same nine putative cyclophilins, this was assumed to represent the complete *S. pombe* cyclophilin repertoire.

The cyclophilins were named by numbering from the smallest to the largest, cyclophilin 1 being the smallest and cyclophilin 9 the largest (Table 1). What is apparent from the information obtained is that they vary both in size and complexity. Surpris-

Table 1
Gene and protein information for the *S. pombe* cyclophilins

GenBank Accession #	Name	Chromosome	Predicted gene size	Predicted coding region	No. of introns	Molecular weight (kDa)	Domains
T38930	Cyclophilin-1	1	899	468	5	16.9	PPIASE
P18253	Cyclophilin-2	2	489	489	0	17.4	PPIASE
T39632	Cyclophilin-3	2	838	522	5	18.9	PPIASE
T40819	Cyclophilin-4	2	699	606	1	22.2	PPIASE, N-terminal LS
S62327	Cyclophilin-5	1	1071	1071	0	40.2	N-term. PPIASE, C-term. TPR
T39728	Cyclophilin-6	2	1299	1299	0	50.8	N-term. PPIASE, central RRM
T39621	Cyclophilin-7	2	1564	1392	3	52.2	N-term. PPIASE
T38137	Cyclophilin-8	1	1700	1416	3	53.6	C-term. PPIASE, N-term. U-Box
T41399	Cyclophilin-9	3	1888	1833	1	69.0	C-term. PPIASE, N-term. WD40 rpts

Five genes are located on chromosome 2 with three on chromosome 1 and one on chromosome 3. The size of the gene is not necessarily indicative of intron number with the smallest having the most introns. Domain key: RRM, RNA recognition motif; U-box, a modified Ring Finger motif that is used in protein chaperoning; TPR, tetratricopeptide repeat used in heat shock protein binding; LS, localisation sequence; WD40, repeat involved in protein–protein interactions.

ingly cyclophilin 1 has the most exons, yet is the smallest gene, whereas cyclophilin 9, the largest, has just two exons. Size does not necessarily correlate with exon complexity in this family. Fig. 1 shows an alignment of the identified *S. pombe* cyclophilins. From this we can see some of the structural characteristics that define some of the cyclophilins as different. SpCyp3 and SpCyp5 have an insert of hydrophilic residues within ruler positions 490–501 that the other cyclophilins do not possess, although SpCyp5's is slightly shorter in length. SpCyp6 has three inserted sequences that do not occur in any of the other cyclophilins and these are seen at ruler positions 542–547, 572–578 and an N at 589. SpCyp7 has an inserted T at ruler position 607 and interestingly is lacking an xLD motif at ruler position 592–594, which is a motif found in all cyclophilins [2]. It does possess an ELD at ruler position 618–620 but whether this does function as the xLD motif and whether it can in fact loop back into the same position in the protein as the xLD normally occupies is unknown.

BLASTP searching of the NCBI database [25] using the putative cyclophilin protein sequences as probes confirmed that they were cyclophilins and identified any orthologous proteins in other species. Table 2 shows the identified orthologues of the *S. pombe* cyclophilins from *Homo sapiens*, *Saccharomyces cerevisiae*, *Drosophila melanogaster*, *Caenorhabditis elegans* and *Mus musculus*.

Cyclophilin 3 (SpCyp3) was identified as the *S. pombe* orthologue of human USA-CyP showing 62% amino acid identity and 78% similarity with this protein. Orthologues were also found in other studied species with some having been well characterised. An alignment of the homologues of SpCyp3 is shown in Fig. 2. In addition to the orthologue in humans (Accession # AF016371), *D. melanogaster* (AE003789), *C. elegans* (CEU34955) and *M. musculus* (NP_006338), an identified orthologue in *Echinococcus multilocularis* (AJ292367), is included. This human parasitic Cestode, also called the fox tapeworm, contains a protein that showed the highest similarity to the *S. pombe* sequence of the USA-CyP sequences currently available.

The alignment shows that the key residues involved in the PPIase catalytic site are highly conserved in SpCyp3 and its homologues. Of interest is

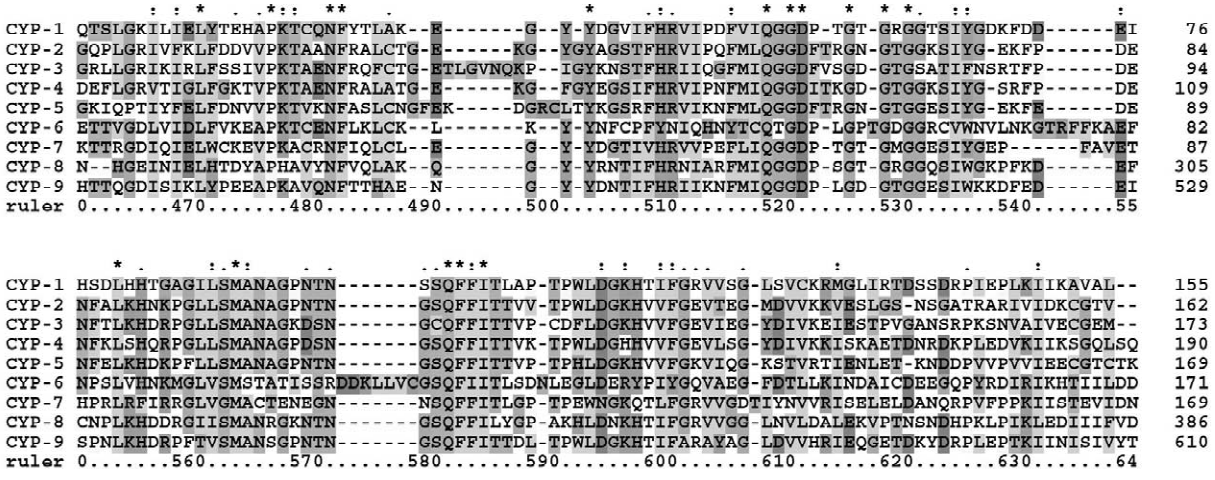

Fig. 1. Alignment of the PPIase domain of the cyclophilins of *Schizosaccharomyces pombe*. '*' indicates positions that have a single, fully conserved residue. ':' indicates that one of the following 'strong' groups is fully conserved: STA, NEQK, NDEQ, QHRK, MILV, MILF, HY, FYW. '.' Indicates that one of the following 'weaker' groups is fully conserved: CSA, ATV, SAG, STNK, STPA, SGND, SNDEQK, NDEQHK, NEQHRK, FVLIM, HFY. These are all the positively scoring groups that occur in the Gonnet Pam250 matrix. The strong and weak groups are defined as strong score >0.5 and weak score ≤ 0.5, respectively. Shading is assigned with relation to the level of consensus within a region that contains a certain sub-set of amino acids. Regions with the same shading are more conserved than those that have different colours. Ruler shows the distance of the amino acid from the beginning of the alignment and is for reference only. Alignment was produced using the ClustalX alignment program [36].

Table 2
Orthologues of the *S. pombe* cyclophilins

S. pombe	*H. sapiens*	*M. musculus*	*D. melanogaster*	*C. elegans*	*S. cerevisiae*
Cyclophilin-1	CGI-124	ND	CG13892	C34D4	ND
Cyclophilin-2	Cyp-A	Cyp-A	Cyp-1	Cyp-3	CPR1p
Cyclophilin-3	USA-Cyp-20	AK003179	CG17266	Cyp-11	ND
Cyclophilin-4	Cyp-B	CyP-S1	CG2852	Cyp-5	Cyp5p
Cyclophilin-5	Cyp-40(D)	TC200669	ND	ND	CPR6p
Cyclophilin-6	AAH20986	BAB28194	AAF56342	NP_492343	ND
Cyclophilin-7	Antigen NY-CO-10	ND	AAF50009	NP_496562	cwc27p
Cyclophilin-8	Cyp-60	TC186889	CG7747	Cyp-4	ND
Cyclophilin-9	HAL539	ND	NP_611935	NP_493378	ND

The orthologues were identified using BLASTP searching of the NCBI database using the *S. pombe* cyclophilins as probes. Amino acid sequences were compared for conserved regions other than the PPIase domain to indicate possible conserved function. ''ND'' indicates that no orthologues of this *S. pombe* cyclophilin could be detected in the published genomic sequences of *D. melanogaster*, *C. elegans* and *S. cerevisiae* or in the *H. sapiens* and *M. musculus* sequences available as of 1 June 2002.

the xLD motif seen at ruler positions 142–144. Two versions of this motif are seen in this alignment, WLD (Human, *M. musculus*) and FLD (*S. pombe*, *C. elegans*, *D. melanogaster* and *E. multilocularis*). Work on human cyclophilin A has shown that the substitution of tryptophan with phenylalanine correlates with a decrease in sensitivity to CsA [26]. We can therefore predict that SpCyp3 will have a decreased sensitivity to CsA when compared with hUSA-CyP. Fig. 3 shows a computer-generated comparison of the hUSA-CyP and SpCyp3 active sites. The structure of hUSA-CyP has been resolved by X-ray crystallography [27] and this structure was used as a template for the generation of the SpCyp3 model using a threading algorithm. The models show the predicted changes occurring around the xLD motif with a F/W substitution that could account for the decrease in the binding affinity of CsA.

In addition to the PPIase domain, the proteins show a high level of conservation in their C-terminus, as observed by Horowitz et al. [21]. The last 40 amino acids of the *S. pombe* cyclophilin 3 show 37.5% identity and 87.5% similarity overall with the human, mouse, *D. melanogaster*, *C. elegans* and *E. multilocularis* orthologues. A well conserved CGEM motif is seen at the C-terminal end that is not seen in any other cyclophilin group. This region contains beta structure (ruler positions 178–185 in Fig. 2) and loops. It stretches from a position near the catalytic arginine in a loop region (ruler positions 168–173 in

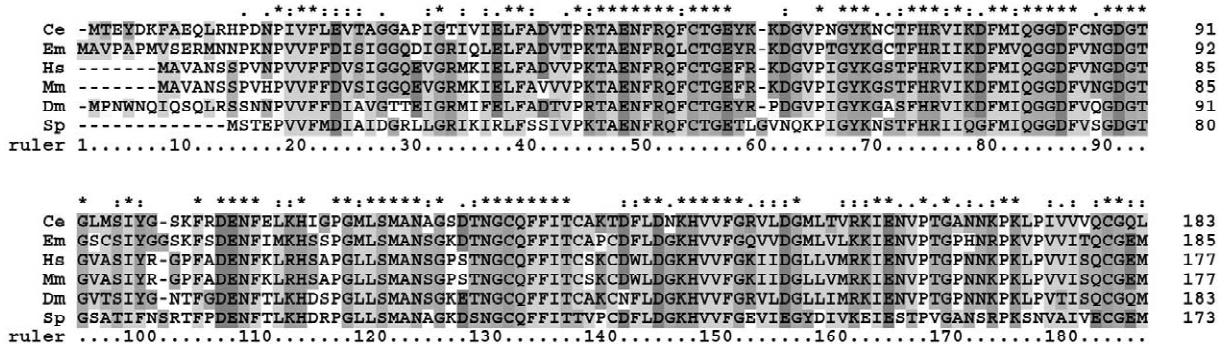

Fig. 2. Comparison of the *S. pombe* cyclophilin 3 sequence with other 20×10^3 rel. mol. mass cyclophilin orthologues. For an explanation of the symbols used, see Fig. 1 legend. The PPIase domain stretches from ruler position 46 to 151. Residues of interest are the xLD motif at ruler position 142–144 and the catalytic arginine residue [37] at ruler position 75. All show homology to each other and the C-terminal region shows a high level of conservation inferring it could have a role in the cyclophilin's function.

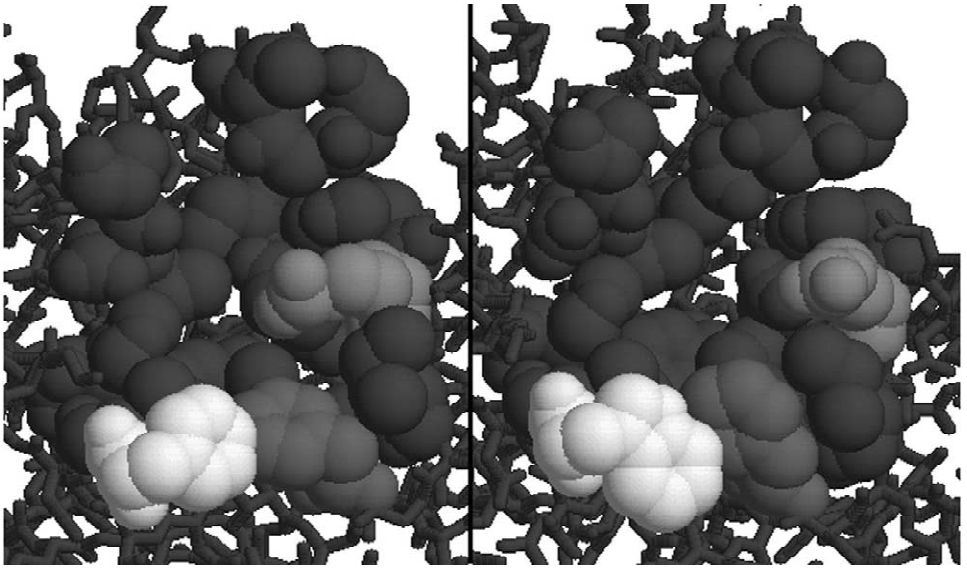

Fig. 3. Structural comparison of the cyclosporin binding domains of human USA-CyP and *S. pombe* cyclophilin 3. Images are of the active site of hUSA-CyP (right) and SpCyp3 (left). The 'x' residue in the xLD motif is coloured white, the catalytic arginine coloured light grey and a phenylalanine that undergoes a positional change due to the change in 'x' coloured in medium grey (ruler position 84 in Fig. 2). The active site changes are clearly visible with the catalytic arginine being brought further into the cleft and the affected phenylalanine rotating to show a larger surface area to the binding molecule with the presence of the F at the 'x' position. The structure of hUSA-CyP was determined by X-ray crystallography [27] and SpCyp3 using a threading algorithm with hUSA-CyP structure as a template [figure produced using RasWin molecular graphics program, version 2.6 (by Roger Sayle). hUSA-CyP PDB file, 1QOI, was obtained from the protein data bank (PDB) maintained by RCSB. SpCyp3 structural file was produced by the Swiss-Model program on the ExPASy Molecular Biology Server].

Fig. 2) with the lysine/arginine at position 173 adjacent to catalytic arginine. The β-strands form a β-sheet with two β-strands (ruler positions 19–26 and 29–38 in Fig. 2) in the N-terminus of the protein. Although the N-terminus is fairly divergent, some residue groups do show conservation and these are in positions within the β-sheet. As this structure is conserved within SpCyp3 and its homologues it could be involved in interactions that define this cyclophilin's role within the cell.

2.2. Cloning of H. sapiens USA-CyP and S. pombe cyclophilin 3

From the EST sequences obtained from the NCBI database the PCR primers 5′-CC**CATATG**-GCGGTGGCAAATTCAAGT-3′ and 5′-CC**CTC-GAG**CATCTCCCCACACTGCGAG-3′ were designed to amplify the human USA-CyP sequence and the primers 5′-CC**CATATG**AGCACCGAA-CCTGTTG-3′ and 5′-CC**CTCGAG**CATTTCT-CCACATTCGACTATC-3′ were designed to amplify the coding region of *S. pombe* cyclophilin 3, as shown in Fig. 4. NdeI and XhoI sites (shown in bold) were incorporated into the primers so that the resulting PCR product could be cloned into pET21a with the coding regions in-frame with a C-terminal His tag. The ''reverse'' primer was designed such that the native stop codon would be removed to allow for C-terminal tagging.

S. pombe genomic DNA was prepared [28] from a culture of SP011 cells (leu$^-$, ade$^-$, ura$^-$), a ''wild-type'' strain of *S. pombe*. Dr L. Hashemzadeh-Bonehi (University of Sussex) kindly provided the *S. pombe* cDNA library. Human cyclophilin 20 was amplified from human prostate cDNA (Clontech, Basingstoke, UK).

The genes were PCR amplified from SP011 genomic DNA or cDNA using the Expand™ High Fidelity PCR system (Roche Molecular Biochemi-

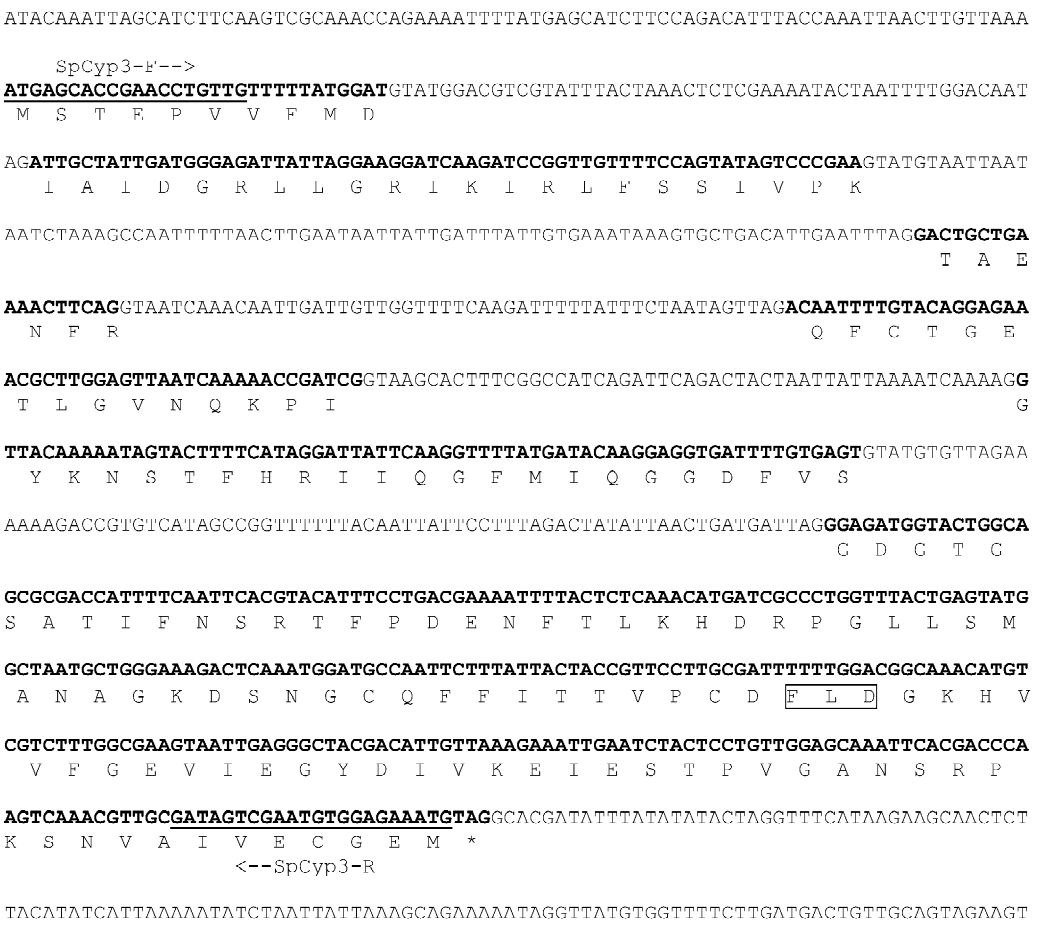

Fig. 4. Gene structure of SpCyp3. The genomic DNA sequence was obtained from the NCBI database using the SPBC1709.04c *S. pombe* cosmid and confirmed by clone sequencing. Exons are shown in bold type with the corresponding amino acid sequence shown below the central character in each codon. The underlined sequence shows the location of the primer sequences and the xLD motif is boxed.

cals) with the primers designed above. The reactions were initially held at 94 °C for 5 min and then run for 30 cycles of 94 °C for 1 min, 50 °C for 1 min and 68 °C for 2 min in a Perkin-Elmer 2400 thermal cycler. The PCR products were then cloned using the TOPO-TA cloning kit (Invitrogen, Groningen, The Netherlands) into the pCR2.1 vector, and shuttled into pET21a using the incorporated restriction sites, such that a C-terminal 6xHis-tag was added to the protein. Clones were sequenced at Genpak (Science Park Square, University of Sussex) using vector specific pET-T7P (TAATACGACTCACTATAGGG) and pET-T7T (GCTAGTTATTGCTCAGCGG) primers to confirm that they coded for the correct protein.

2.3. Production of recombinant protein

Expression was carried out in BL21(DE3) cells (Novagen, Madison, WI, USA); 100 ml cultures were grown at 37 °C to an $O.D._{600}$ of 0.6–0.8 in 2YT medium before induction of expression with 1 μ*M* IPTG (Sigma, Poole, UK). Cells were harvested by centrifugation 3 h post-induction. The His-tagged protein was purified from sonicated cell lysates using TALON immobilised metal affinity resin (Clontech, Basingstoke, UK) according to the manufacturer's instructions. Purification was then assessed using SDS–PAGE and Western blotting with a rabbit anti-His primary antibody (Pfizer, Sandwich, UK), and alkaline phosphatase conjugated mouse anti-rabbit

secondary antibody (Sigma, Poole, UK). Fig. 5 shows the analysis of SpCyp3 expression and purification.

2.4. PPIase assays

PPIase activity assays were carried out according to the methods of Fisher et al. [29]. Reactions were carried out at 10 °C in 50 m*M* Hepes, pH 7.8 (Sigma, Poole, UK), containing 25 m*M* substrate. The cyclophilin was added at the same time as the reaction was started by the addition of chymotrypsin (Sigma) to a final concentration of 250 μg/ml. Following mixing, absorbance at 390 nm was recorded for 2.5 min using a UV3 spectrophotometer (Unicam, Cambridge, UK) and Unicam Vision Software.

Four tetrapeptide substrates (Suc–Ala–Xxx–Pro–Phe–pNA, where Xxx is either Ala, Glu, Phe or Leu) were obtained from Bachem (Saffron Walden, UK) and tested against the cyclophilins to see if they possessed any difference in substrate specificity (data not shown). We found both had the same substrate preferences with preferential activity on the tetrapeptide containing alanine. The tetrapeptide containing the alanine was used in all the subsequent reactions.

Cyclosporin A was obtained from Sandoz Pharmaceuticals (Basel, Switzerland). FK506 was obtained from Fujisawa Pharmaceuticals (Osaka, Japan), and rapamycin from Wyeth-Ayerst Research Laboratories (Princeton, NJ, USA). Both cyclophilins were unaffected by 5 μ*M* FK506 and 5 μ*M* rapamycin treatment (data not shown), but showed sensitivity to 5 μ*M* CsA as seen in Fig. 6. CsA only inhibits the cyclophilin family whereas FK506 [4] and rapamycin [30] only inhibit the FKBPs (FK506 Binding Protein PPIases). These results demonstrate that the PPIase activity was specifically cyclophilin activity.

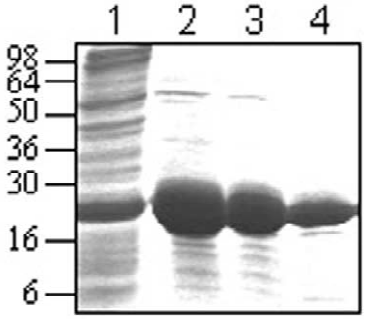

Fig. 5. Purification of *S. pombe* cyclophilin 3 from *E. coli* cell lysates by TALON bead resin. Lane 1: Soluble protein extract, 3 h post-induction; lanes 2–4: 10-μl samples from sequential 0.5 ml imidazole elution fractions. Numbers to the left of the gels are the molecular masses in 10^3 rel. mol. mass units.

To calculate the K_i of the cyclophilins for CsA and their k_{cat}/K_M values, PPIase activity data were fitted to a first-order rate equation with offset ($A_t = A_\alpha\ (1-e^{-kt})+\text{offset}$) using the GraFit package (Erithacus Software Ltd). This yield a value '*k*' that is used to calculate k_{cat}/K_M values as shown by Liu et al. [26]. The relationship between the velocity ratio, v_i/v_o *v*. [I], for a tight binding inhibitor is described by the Holt tight binding equation [31]. The equation was written into the Grafit custom equation file and a three-dimensional fit carried out (using v_i/v_o, [I] and [E] as variables) to yield an estimate for K_i.

Both SpCyp3 and hUSA-CyP had comparable PPIase activity with the succinyl–Ala–Ala–Pro–Phe–pNA substrate under standard conditions with k_{cat}/K_M values approximately 1.5 $\mu M^{-1}\ s^{-1}$. Interestingly, however, their K_i values for CsA differed by more than an order of magnitude. hUSA-CyP had a calculated K_i value of 121±37 n*M* and SpCyp3 a value of 2097±582 n*M*. This difference can be attributed to the difference in the xLD motif found in the active site. As we predicted earlier, the change from tryptophan (hUSA-CyP) to phenylalanine (SpCyp3) has caused a decrease in the sensitivity to CsA, which correlates with what was found in human cyclophilin A [26]. A mutated version of hUSA-CyP replacing W with F (hUSA-CyP-W133F) also showed a greater resistance to CsA [23]. The changes in the active site visible in Fig. 3 appear to have caused a weakening in the cyclophilin–CsA interaction, therefore reducing the cyclophilin's affinity for CsA. This would therefore lead to the reduced sensitivity we have seen in our results.

2.5. GFP-Tagging

The localisation of SpCyp3 within the *S. pombe* cell would be expected to be nuclear if it is to share a

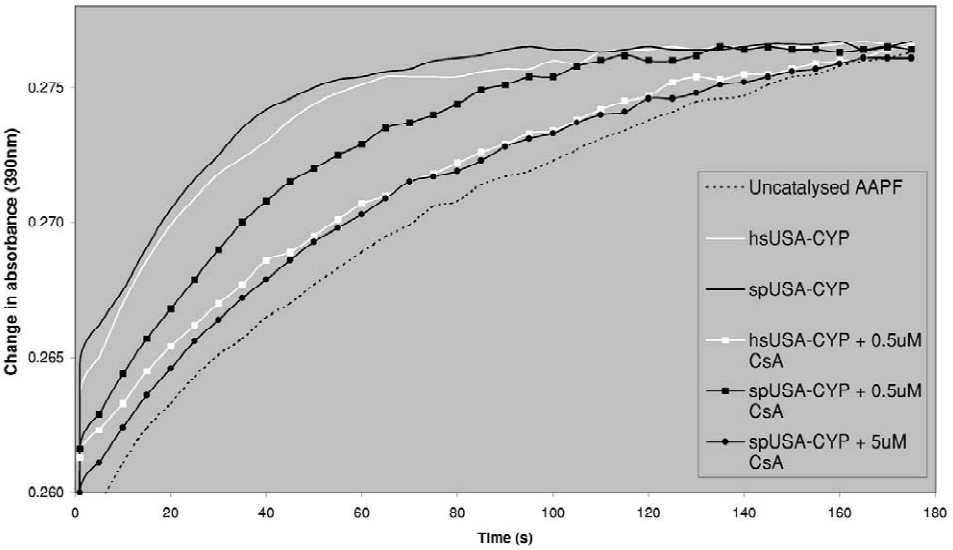

Fig. 6. Chart showing the PPIase activity and cyclosporin A (CsA) sensitivity of human USA-CyP (white lines) and *S. pombe* cyclophilin 3 (black lines) on the tetrapeptide substrate succ-AAPF-pNA. The dashed line shows the rate of spontaneous *cis*/*trans* isomerisation in the absence of a PPIase. Solid lines show the isomerisation catalysed by 10 n*M* cyclophilin. Squares show the catalysed reaction inhibited by the addition of 0.5 μ*M* CsA, and circles show the reaction inhibited by 5 μ*M* CsA.

conserved function with its human orthologue. To investigate this, the *S. pombe* expression vector pREP41-GFP [32] (kindly provided by Dr F. Watts, University of Sussex) was used. The 3′ PCR primers for amplifying the cyclophilin sequences were modified to incorporate a BamHI site instead of an XhoI site. NdeI/BamHI fragments were cloned into pREP41-GFP with the cyclophilin sequences in-frame with an N-terminal GFP tag. Expression constructs were transformed into ''wild-type'' sp011 (leu^-, ade^-, ura^-) strain *S. pombe* and selected by leucine prototrophy. Cultures were grown under leucine auxotrophic selection at 30 °C for 48 h in the absence of thiamine to induce the nmt1 promoter. Live cells were viewed using a Nikon Optiphot-2 microscope with a Bio-Rad MRC600 laser scanning apparatus.

The results of this experiment are shown in Fig. 7(A,C) where a majority of the GFP fluorescence can be seen in the nucleus. SpCyp3 appears to be localised mainly in the nucleus, with the cytoplasmic fluorescence probably due to the over expression, adding evidence to the conserved function of SpCyp3 and hUSA-CyP. The same experiment was then repeated with hUSA-CyP to determine whether it too localised to the nucleus in *S. pombe*. Fig. 7(B,D) confirms that in this case hUSA-CyP also localises to the nucleus. This gives strong support to the idea that hUSA-CyP and SpCyp3 share conserved function within the spliceosome complex of the cell. However, the nuclear localisation was not immediate. The proteins showed cytoplasmic localisation 24 h post-induction, only becoming nuclear 48 h post-induction, indicating it is possibly due to target protein interactions.

3. Discussion

The cyclophilin repertoire of *S. pombe* has been identified using BLASTP searching of the Sanger Centre *S. pombe* database. Their homologues in different species were then found using BLASTP searching of the NCBI database. Human USA-CyP and *S. pombe* cyclophilin 3 were identified as orthologues with 62% amino acid identity over their whole sequence. Alignment analysis with other proposed homologues have shown that the C-terminal region is highly conserved and, along with conserved regions in the N-terminus and an inserted

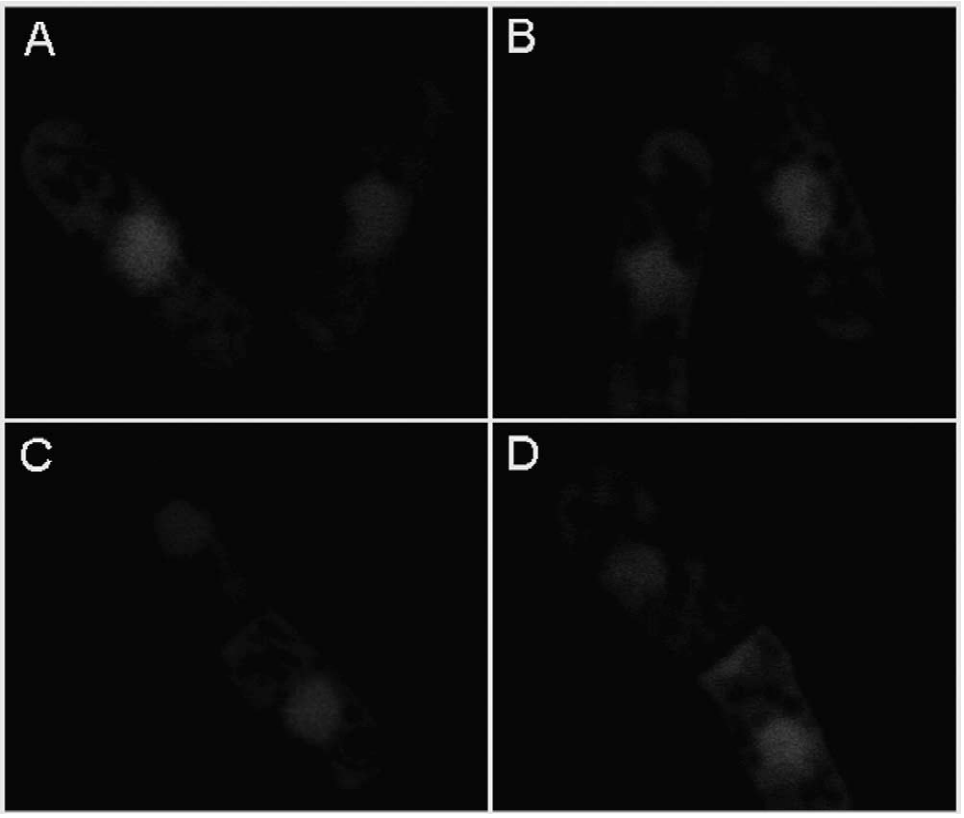

Fig. 7. Expression of GFP-tagged human USA-CyP (B and D) and *S. pombe* cyclophilin 3 (A and C) in ''wild-type'' SP011 *S. pombe* cell. A and B show localisation in *S. pombe* cells in G_1 phase with C and D showing localisation in cells in M phase. No GFP fluorescence was visible in cells transformed with a pREP41-GFP construct containing no insert and induced under the same conditions.

loop in the PPIase domain, might play a role in this cyclophilin's function.

Human USA-CyP has been shown to associate with the pre-mRNA-splicing complex through interactions with hPrp3, hPrp4 [21,22] and hPrp18 [23] proteins that complex and then associate with the U4/U6·U5 snRNPs. Treatment with cyclosporin A has been shown to inhibit the second step of splicing in vitro and cause disruption of splicing in vivo through the interaction of hUSA-CyP and hPrp18 [23]. *S. pombe* does use splicing [19], Prp protein homologues have been found within its genome [23,33,34] and these orthologues possess the 31 amino acid homologous region that has been identified as the USA-CyP binding site [23].

The use of N-terminal GFP tagging of both proteins and subsequent expression in *S. pombe* cells has shown both localising preferentially to the nucleus. The cyclophilin sequences themselves do not appear to have an identifiable nuclear localisation motif and their nuclear accumulation was not immediate but became visible 48 h post-induction. This accumulation could therefore be due to the association of the cyclophilin with native Prp4 and Prp18, which have their own NLS [35].

PPIase assays were carried out to compare the activity of the cyclophilins and their sensitivity to the PPIase inhibiting drugs. Both were unaffected by FK506 and rapamycin, the FKBP inhibitors, but were sensitive to cyclosporin A. Although they shared comparable K_{cat}/K_M values with the tetrapeptide substrate (Suc–Ala–Ala–Pro–Phe–pNA), their inhibition constants differed by more than an order of magnitude. This difference has been attribu-

ted to their difference in the xLD motif in the PPIase active site. The presence in the 'x' position of the phenylalanine (SpCyp3) gave a reduced sensitivity to cyclosporin A compared to the presence of a tryptophan (hUSA-CyP).

4. Conclusions

These results lead us to the conclusion that *S. pombe* cyclophilin 3 and human USA-CyP are orthologues with a conserved function within the spliceosome. Mutagenesis and protein interaction studies coupled with studies into the effect of cyclosporin A on the *S. pombe* splicing process are needed to confirm that this is indeed the case. With the presence of a phenylalanine in the xLD motif of SpCyp3 it is likely that the splicing process will be unaffected by cyclosporin A treatment, as was shown with the hUSA-CyP-W133F mutant [23]. Mutagenesis studies of the binding site identified by Horowitz et al. [23] in the *S. pombe* Prp4 and Prp18 homologues will therefore be of the most interest along with the production of a SpCyp3-F133W mutant to study the effect this has on cyclosporin A sensitivity. This work illustrates how *S. pombe* can be used as a model for the characterisation of fundamental cellular processes such as RNA splicing outside the human cell.

Acknowledgements

Trevor Pemberton is in receipt of a BBSRC Studentship and Stuart Rulten was in receipt of a BBSRC CASE award sponsored by Pfizer Central Research, UK, at the time of this research. The authors would like to thank Dr Ross Kinloch for his cooperation with this project. We would also like to thank Drs Felicity Watts, Nick Warr, Deborah Taylor and Jenny Ho for the provision of *S. pombe* strains and reagents, and for helpful technical advice.

References

[1] S.F. Gothel, M.A. Marahiel, Cell Mol. Life Sci. 55 (1999) 423.
[2] A. Galat, Arch. Biochem. Biophys. 371 (1999) 149.
[3] T. Maruyama, M. Furuani, Front. Biosci. 5 (2000) 821.
[4] S. Ho, N. Clipstone, L. Timmermann, J. Northrop, I. Graef, D. Fiorentino, J. Nourse, G. Crabtree, Clin. Immunol. Immunopathol. 80 (1996) S40.
[5] G. Fischer, B. Wittmann-Liebold, K. Lang, T. Kiefhaber, F.X. Schmid, Nature 337 (1989) 476.
[6] R.E. Handschumacher, M.W. Harding, J. Rice, R.J. Drugge, D.W. Speicher, Science 226 (1984) 544.
[7] J. Kallen, C. Spitzfaden, M.G. Zurini, G. Wider, H. Widmer, K. Wuthrich, M.D. Walkinshaw, Nature 353 (1991) 276.
[8] D. Braaten, E.K. Franke, J. Luban, J. Virol. 70 (1996) 3551.
[9] M.M. Endrich, P. Gehrig, H. Gehring, J. Biol. Chem. 274 (1999) 5326.
[10] E.K. Franke, H.E. Yuan, J. Luban, Nature 372 (1994) 319.
[11] L. Yin, D. Braaten, J. Luban, J. Virol. 72 (1998) 6430.
[12] R.T. Miller, A.P. Anderson, J.C. Corton, R.C. Cattley, Carcinogenesis 21 (2000) 647.
[13] A.A. Duina, H.M. Kalton, R.F. Gaber, J. Biol. Chem. 273 (1998) 18974.
[14] J.P. Steiner, M.A. Connolly, H.L. Valentine, G.S. Hamilton, T.M. Dawson, L. Hester, S.H. Snyder, Nat. Med. 3 (1997) 421.
[15] R.J. Bram, D.T. Hung, P.K. Martin, S.L. Schreiber, G.R. Crabtree, Mol. Cell. Biol. 13 (1993) 4760.
[16] F. Etzkorn, Z. Chang, L. Stolz, C. Walsh, Biochemistry 33 (1994) 2380.
[17] S.L. Schreiber, G.R. Crabtree, Immunol. Today 13 (1992) 136.
[18] C.T. Walsh, L.D. Zydowsky, F.D. McKeon, J. Biol. Chem. 267 (1992) 13115.
[19] V. Wood, P. Nurse et al., Nature 415 (2002) 871.
[20] R.L. Tatusov, E.V. Koonin, D.J. Lipman, Science 278 (1997) 631.
[21] D.S. Horowitz, R. Kobayashi, A.R. Krainer, RNA 3 (1997) 1374.
[22] S. Teigelkamp, T. Achsel, C. Mundt, S.F. Gothel, U. Cronshagen, W.S. Lane, M. Marahiel, R. Luhrmann, RNA 4 (1998) 127.
[23] D.S. Horowitz, E.J. Lee, S.A. Mabon, T. Misteli, EMBO J. 21 (2002) 470.
[24] W. Gish, S. Altschul, M. Webb, E. Myers, D. Lipman, J. Mol. Biol. 215 (1990) 403.
[25] S.F. Altschul, T.L. Madden, A.A. Schaffer, J. Zhang, Z. Zhang, W. Miller, D.J. Lipman, Nucleic Acids Res. 25 (1997) 3389.
[26] J. Liu, C.M. Chen, C.T. Walsh, Biochemistry 30 (1991) 2306.
[27] U. Reidt, K. Reuter, T. Achsel, D. Ingelfinger, R. Luhrmann, R. Ficner, J. Biol. Chem. 275 (2000) 7439.
[28] J. Sambrook, D.W. Russell, Molecular Cloning – A laboratory manual, Cold Spring Harbor Laboratory Press, 3rd ed., Cold Spring Harbor, NY, 2001, p. 4.70.
[29] G. Fischer, H. Bang, C. Mech, Biomed. Biochim. Acta 43 (1984) 1101.
[30] T. Jayaraman, A. Marks, J. Biol. Chem. 268 (1993) 25385.
[31] D. Holt et al., J. Am. Chem. Soc. 115 (1993) 9925.

[32] R. Craven, D.J. Griffiths, K.S. Sheldrick, R.E. Randall, I.M. Hagan, A.M. Carr, Gene 221 (1998) 59.
[33] S. Alahari, H. Schmidt, N. Kaufer, Nucleic Acids Res. 21 (1993) 4079.
[34] L. Ayadi, I. Callebaut, C. Saguez, T. Villa, J.-P. Mornon, J. Banroques, J. Mol. Biol. 284 (1998) 673.
[35] K. Nakai (2000) http://psort.nibb.ac.jp/psort/helpwww2.html
[36] J.D. Thompson, T.J. Gibson, F. Plewniak, F. Jeanmougin, D.G. Higgins, Nucleic Acids Res. 24 (1997) 4876.
[37] E.Z. Eisenmesser, D.A. Bosco, M. Akke, D. Kern, Science 295 (2002) 1520.