Cyclophilin sensitivity to sanglifehrin A can be correlated to the same specific tryptophan residue as cyclosporin A

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Abstract Sanglifehrin A (SFA) is a recently discovered immunosuppressant drug that shares its intracellular target with the major immunosuppressant drug cyclosporin A (CsA). Both bind to and inhibit the cyclophilins, a diverse family of proteins found throughout nature that share a conserved catalytic domain. Although they share this common protein target, the mechanism of action of the cyclophilin–SFA complex has been reported as distinct from that of the well-studied cyclophilin–CsA complex. The X-ray structure of a macrolide analogue of SFA's cyclic region complexed with cyclophilin A has recently been resolved, but this left the placement of the linear region of SFA unresolved. Using five cyclophilins from the fission yeast *Schizosaccharomyces pombe*, and a mutant of one of these proteins, SpCyp3-F128W, we have shown that the sensitivity of cyclophilins to SFA can be correlated to the same specific tryptophan residue that has previously been identified to correlate to CsA sensitivity, and that the tail of SFA may be responsible for mediating this sensitivity.

Key words: Cyclophilin; Peptidyl prolyl *cis*/*trans* isomerase; Cyclosporin A; Sanglifehrin A; *Schizosaccharomyces pombe*

1. Introduction

Cyclosporin A (CsA) is an important immunosuppressive drug whose activity requires an interaction with a cyclophilin [1]. Immunosuppression is caused by a cyclophilin–CsA complex inhibiting the Ca^{2+}-dependent protein phosphatase calcineurin [1–3]. Proliferation of activated T-cells is dependent upon the expression of the cytokine interleukin-2 [4,5]. Interleukin-2 transcription is activated by the transcription factor nuclear factor of activated T-cell, 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 T-cell proliferation and therefore immunosuppression.

Sanglifehrin A (SFA) is a new immunosuppressant drug. Shown to interact with human cyclophilin A [6] and more recently mitochondrial cyclophilin D [7], it has a novel mechanism of immunosuppression that is distinct from CsA. SFA blocks cell cycle progression from G_1 through the nuclear factor (NF)-κB-dependent activation of p53 [8,9], but independent of the calcineurin-dependent deactivation of the NF-κB inhibitor IκB/MAD3 [10]. Although the mechanism is not yet fully understood, the cyclophilin–SFA complex again binds to an intracellular protein that leads to the overproduction of p53.

In early 2002 the genome of the fission yeast *Schizosaccharomyces pombe* was completed by the sequencing consortium led by the Sanger Centre [11] and we have previously reported the identification of nine cyclophilins, of varying complexity, that are encoded in the *S. pombe* genome [12]. We report here the cloning and expression of five cyclophilins from *S. pombe* (SpCyp1–5) with drug inhibition assays and tryptophan fluorescence titrations showing that the sensitivity of the cyclophilins to the immunosuppressant drugs CsA and SFA can be correlated to the same specific tryptophan residue.

2. Materials and methods

2.1. Cloning, expression and purification of SpCyp1–5

SpCyp1–5 were cloned, expressed and purified as previously described for SpCyp3 [12]. SpCyp2 and SpCyp5 were cloned from genomic DNA as they contain no introns, with SpCyp1 and SpCyp4 cloned from cDNA as they possess five introns and one intron respectively [12]. SpCyp1 was polymerase chain reaction (PCR) amplified from 2 μl of cDNA produced by reverse transcription (RT)-PCR using the enhanced avian RT-PCR kit (Sigma, Poole, UK) from RNA purified from *S. pombe* cells using the RNeasy midi kit (Qiagen, Hilden, Germany). SpCyp4 was cloned without its N-terminal signal peptide as it proved insoluble when this was present, which mirrors what was seen by Price et al. during their expression of its human orthologue cyclophilin B [13]. All cyclophilins were expressed from the pET21a expression system (Novagen, WI, USA), which gave them an in-frame C-terminal $(His)_6$ fusion tag, using the conditions previously described by us [12] except for SpCyp1 which proved insoluble using these conditions and was subsequently expressed at room temperature and induced with 0.5 μM isopropyl thiogalactose (IPTG). Purification was then achieved using the TALON Cell-Thru IMAC resin from BD Biosciences (Oxford, UK) as per the manufacturers' instructions.

2.2. PCR mutagenesis of SpCyp3

Two overlapping 30-mer primers were designed so that the phenylalanine to tryptophan mutations (in bold) lay at their centre (5′→3′: CGTTCCTTGCGAT**TGG**TTGGACGGCAAAC, 3′→5′: GTTTGCCGTCCAA**CCA**ATCGCAAGGAACG). 50 μl reactions were prepared containing 0.5 μl of a Qiagen miniprep (Hilden, Germany) of the pET21a construct of the native gene, 2.5 pM of each primer, 2 μl of a 10 mM deoxyribonucleoside triphosphate (dNTP) mix (Invitrogen, The Netherlands), 1 μl of Pfu DNA polymerase (Stratagene, UK) and 5 μl of 10× Pfu buffer. PCR was carried out in a Perkin-Elmer GeneAmp PCR system 2400 thermocycler and cycled as follows: 94°C for 5 min (initial denaturing step), followed by 20 cycles of 94°C for

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Abbreviations: CsA, cyclosporin A; SFA, sanglifehrin A; SpCyp, *Schizosaccharomyces pombe* cyclophilin; PPIase, peptidyl-prolyl *cis*/*trans* isomerase

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1 min (denaturing), 55°C for 1 min (annealing) and 68°C for 13 min (extension). A final 68°C for 7 min incubation was included before placing the reactions at 4°C. 1 μl of the restriction endonuclease *Dpn*I (Promega, Southampton, UK) was added to remove template plasmid DNA in a 1 h incubation at 37°C followed by a 15 min incubation at 65°C to inactivate the enzyme. 10 μl of the reaction was then transformed into chemically competent TOP-10 *Escherichia coli* cells and plated onto Luria–Bertani (LB) plates containing 50 μg/ml ampicillin and grown overnight at 37°C. Colonies were selected at random, cultured in 10 ml LB media containing 50 μg/ml ampicillin, Qiagen miniprep (Hilden, Germany) and sent for sequencing to Oswel Research Products (Southampton, UK) to confirm their identity prior to expression as previously described [12].

2.3. *Peptidyl-prolyl* cis/trans *isomerase (PPIase) assays and drug inhibition*

The SpCyp1–5 all showed strong PPIase activity with the synthetic Ala-Pro peptide (*N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanalide (succ-AAPA-pNA); Sigma, Poole, UK) as a substrate compared to other peptides tested and thus all the prolyl isomerase activities of the recombinant $(His)_6$ fusion tagged cyclophilins were analysed with this substrate using a previously published method [14] based on the original method by Fischer et al. [15], except for SpCyp5 which used a variation of this assay which replaces the protease chymotrypsin with subtilisin [16] as it showed sensitivity to chymotrypsin (data not shown). Reactions were performed in triplicate at 10°C with measurements taken at 390 nm using a Unicam UV3 spectrophotometer (Cambridge, UK) for 150 s as previous investigations with other PPIases (data not shown) had shown no effect on subsequent calculations when reactions were monitored for longer. CsA (Sandoz Pharmaceuticals, Basel, Switzerland) was used at concentrations of 1 nM–10 μM and SFA (kind gift of Dr. Richard Sedrani, Novartis Pharma AG, Switzerland) was used at concentrations of 1 pM–1 μM. Protein stock concentrations were calculated using the Bradford assay (Bio-Rad, Hercules, CA, USA) which was used in calculating the assay concentration of protein. k_{cat}/K_m values were calculated as previously described [17] and K_i values were calculated as previously described [18] using the GraFit software package (Erithacus Software Ltd).

2.4. *Fluorescence spectroscopy*

The effect of drug binding on the fluorescence of the tryptophan residue present in the xLD motif of the active site of most of the cyclophilins was assessed as previously described by Liu et al. [17] using a Varian Cary Eclipse fluorescence spectrophotometer (Palo Alto, CA, USA). CsA was titrated between 0.001 and 1000 nM and SFA between 0.001 and 1000 pM as these seemed appropriate ranges given the inhibition constants seen with the cyclophilins. Titrations were performed with 50 nM cyclophilin, one sixth of that used by Liu et al., as this was the lowest concentration of cyclophilin that gave a reliably detectable and accurate signal on the equipment used. Each data set was fitted in SigmaPlot version 8.03 (SPSS Inc.; Chicago, IL, USA) using the equation previously published by Veitch et al. ([19]; eq. 1) in a plot of fluorescence change against drug concentration.

3. Results

We have previously reported the identification of the nine SpCyps [12]. We have now cloned, expressed and purified five of these, SpCyp1–5, that all have probable human orthologues. All of the cyclophilins gave the expected PCR band sizes from both genomic and complementary DNA confirmed by sequencing after subcloning and prior to expression. Purification was confirmed using sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blotting using a $(His)_6$ fusion tag antibody (Pfizer, Sandwich, UK). The purified cyclophilin proteins can be seen in Fig. 1.

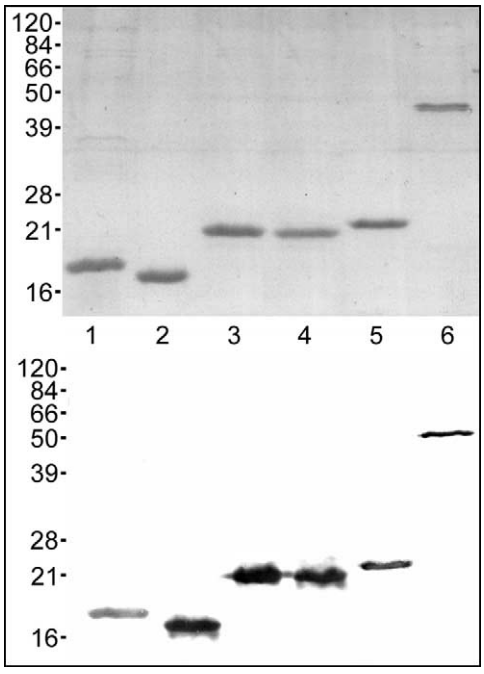

Fig. 1. 15% SDS–PAGE gel and alkaline phosphatase Western blot of the purified SpCyps. SpCyp1 (lane 1), SpCyp2 (lane 2), SpCyp3 (lane 3), SpCyp3-F128W mutant (lane 4), SpCyp4 (lane 5) and SpCyp5 (lane 6). SpCyp1, SpCyp2 and SpCyp4 ran at their predicted M_w of 17.4, 16.9 and 22.2 kDa respectively. SpCyp3 and its F128W mutant ran larger than their predicted 18.9 kDa at about 21 kDa, which can be explained by their p*I* of 5.61. SpCyp5 ran larger than the predicted 40.2 kDa, at about 50 kDa, which cannot be explained by its p*I* of 8.05. Molecular weight marker sizes are shown down the left hand side of the images in kDa.

3.1. *PPIase assays*

Prolyl isomerase assays were carried out as described above using the purified $(His)_6$ fusion tagged proteins. X-ray crystallography studies on orthologues of the SpCyps have shown that the C-termini of the proteins are located on the opposite side to the active site (SpCyp2–4; [20–22] respectively), or in the case of SpCyp5 in the non-PPIase C-terminal domain [23]. Any small C-terminal fusion tag, such as the $(His)_6$ tag used, should therefore not affect substrate or drug binding.

Table 1
The catalytic efficiencies (k_{cat}/K_m) and drug inhibition constants (K_i) of the purified SpCyps

	xLD	k_{cat}/K_m (μM^{-1} s^{-1})	K_i CsA (nM)	K_i SFA (pM)
SpCyp1	WLD	2.0	27 ± 8.2	3.5 ± 0.5
SpCyp2	WLD	11.0	26 ± 5.2	1.2 ± 0.5
SpCyp3	FLD	11.0	1800 ± 450	450 ± 140
SpCyp4	WLD	8.8	15 ± 3.5	1.7 ± 0.2
SpCyp5	HLD	4.1	420 ± 200	130 ± 51
SpCyp3-F128W	WLD	11.0	14 ± 5.0	0.2 ± 0.1

k_{cat}/K_m given for the succ-AAPF-pNA tetrapeptide substrate (μM^{-1} s^{-1}; 2 s.f.) and drug inhibition constants (2 s.f. ± error) for both CsA (nM) and SFA (pM). Errors stated were produced by the Grafit programme when calculating the constant and thus represent its confidence level.

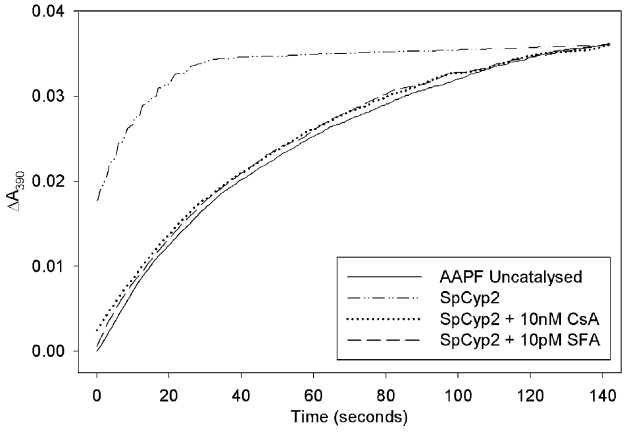

Fig. 2. Effect of cyclophilin inhibiting drugs on the activity of SpCyp2. Plots of change in absorbance against assay time in the presence and absence of the two inhibiting drugs, CsA and SFA, obtained using the PPIase spectrophotometric assay.

Table 1 shows the catalytic efficiencies (k_{cat}/K_m) of the cyclophilins with the succ-AAPF-pNA substrate and their inhibition constants (K_i) with both CsA and SFA. An example graph illustrating the effect of the two inhibitory drugs on the catalytic activity of SpCyp2, seen using the PPIase spectrophotometric assay, and subsequently used in k_{cat}/K_m and K_i calculations, can be found in Fig. 2.

The cyclophilins show a range of activities on the succ-AAPA-pNA tetrapeptide substrate. SpCyp2 and SpCyp3 show the greatest activity with k_{cat}/K_m values of 11.3 and 11.2 μM^{-1} s^{-1} respectively. These are close to the values of 14.6 [14], 16 [13] and 17 [17] μM^{-1} s^{-1} reported for human cyclophilin A in three separate studies and 11 μM^{-1} s^{-1} reported for *Zea mays* cyclophilin A [24]. SpCyp4 showed an activity of 8.79 μM^{-1} s^{-1}, comparable to the activity reported for human cyclophilin B [13] and *Orpinomyces* sp. cyclophilin B [25], which showed 6.3 and 9.3 μM^{-1} s^{-1} respectively, but only about a third of the 25 μM^{-1} s^{-1} reported for *Z. mays* cyclophilin B [24]. SpCyp5 showed an activity of 0.27 μM^{-1} s^{-1} when using chymotrypsin in the assay, which is comparable to the values reported for its *Saccharomyces cerevisiae* orthologues Cpr6, which gave 0.48 [26] and 0.5 [27] μM^{-1} s^{-1} and Cpr7 with 0.08 μM^{-1} s^{-1} [26]. This activity increased to 4.14 μM^{-1} s^{-1} when the assays were repeated with subtilisin after SpCyp5 was found to be sensitive to chymotrypsin (data not shown), showing that the degradation did have an effect on the protein activity and may also explain the low activities seen with its *S. cerevisiae* orthologues if they share this sensitivity to chymotrypsin. The lowest activity was seen with SpCyp1, which showed an activity of 1.98 μM^{-1} s^{-1}. This is 33 times that of the lowest currently reported activity, which was that of *Drosophila melanogaster* Moca-CyP with a rate of 0.06 μM^{-1} s^{-1} [28].

The inhibition constants (K_i) on CsA show that SpCyp1 and SpCyp2 have about the same sensitivity with K_i's of around 27 nM. These values are higher than the 2.6 nM reported previously for human cyclophilin A [29] and the 6 nM reported with *Z. mays* cyclophilins A and B [24]. SpCyp4 is the most sensitive with a slightly lower K_i of 14.6 nM, but this is still higher than the lowest observed K_i's which were seen with *Brugia malayi* cyclophilins 1 and 2 of less than 0.5 nM [30]. SpCyp3 and SpCyp5 gave K_i's of 1762 and 423 nM respectively, which are markedly higher than those of SpCyp1, SpCyp2 and SpCyp4.

This reduction in drug sensitivity can be correlated, as previously reported [17,31], to a variation in the residue found in the 'x' position of the xLD motif located in the active site of the cyclophilins [12]. The presence of a tryptophan in this position, as is found in SpCyp1, SpCyp2 and SpCyp4, confers the greatest sensitivity whereas the presence of a phenylalanine, as is found in SpCyp3, confers the least sensitivity with the presence of a histidine, as is found in SpCyp5, conferring a sensitivity between that of tryptophan and phenylalanine. This result shows the same pattern seen when Liu et al. compared the presence of a tryptophan or phenylalanine in both *E. coli* and human cyclophilin A [17] and Hoffmann et al. compared the presence of a tryptophan in place of the wild-type histidine in human cyclophilin 40 [31].

The most striking feature of the inhibition constants seen with SFA, besides its greater potency which has been previously reported [6], is that they follow the same pattern of drug sensitivity than that is seen with CsA with regards to the xLD isoforms. SpCyp2 and SpCyp4 show similar K_i values of about 1.5 pM with SpCyp1 showing a slightly higher K_i of 3.53 pM. All possess a tryptophan in the xLD motif, with conservation seen at all other positions that contact CsA except for the two variations seen in SpCyp1 which appear not to cause any major effect. The presence of a phenylalanine in the xLD motif (SpCyp3) again causes an order of magnitude greater insensitivity to SFA in a ratio close to that seen with CsA. The presence of a histidine (SpCyp5) again gives a K_i that is in between those that possess a tryptophan or phenylalanine. It is important to note, however, that although the same pattern of sensitivity is seen with regards to the xLD isoform, the cyclophilins possessing a tryptophan vary in sensitivity differently to that seen with CsA, inferring that some different residues in the active site are also involved in conferring SFA sensitivity, but their effect is less pronounced.

3.2. Cyclophilin 3 mutagenesis

To confirm that the drug sensitivity pattern seen with SFA, which appears to correlate to the specific tryptophan residue in the xLD motif, is in fact due to this residue, a mutant SpCyp3 was constructed using PCR mutagenesis replacing the wild-type phenylalanine with tryptophan to produce an SpCyp3-F128W protein. After sequencing confirmed that the engineered mutation was the only deviation from wild-type, it was expressed as previously described for cyclophilin 3 [12] and its purification confirmed using SDS–PAGE and Western blotting using a $(His)_6$ fusion tag antibody (Pfizer, Sandwich, UK). The purified protein can be seen in lane 4 of Fig. 1. Catalytic efficiency and drug sensitivity assays were then carried out and the results shown in Table 1.

The drug inhibition assays showed that the sensitivity of the SpCyp3-F128W mutant to both CsA and SFA is an order of magnitude greater than that of the wild-type protein to both inhibitors whilst PPIase activity assays show that the protein's catalytic activity has not been significantly altered. The wild-type protein sensitivity to CsA was 1762.64 nM compared to the mutant sensitivity (13.45 nM) and the SFA sensitivities were 451.64 pM compared to 0.23 pM respectively. Comparing these to the sensitivities of the other proteins investigated in this study, SpCyp3-F128W CsA sensitivity of 13.45 nM is approximately the same as that of cyclophilin 4 which gave a

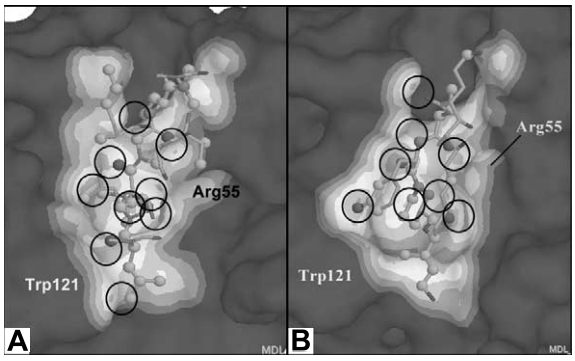

Fig. 3. Molecular graphics comparison of the binding of CsA and SFA. (A) shows CsA [33] and (B) shows macrolide 16 [32], an analogue of SFA's cyclic region that binds to the cyclophilin, bound to the active site of human cyclophilin A. Images were produced using Protein Explorer [34] with the contact surface between the protein and ligand predicted and points of theoretical contact marked by the rings.

value of 14.56 nM, the lowest seen in the wild-type proteins, and which also possesses a tryptophan at its 'x' position. Its SFA sensitivity is the highest we have observed from these six proteins, with a K_i value of 0.23 pM.

3.3. Comparison of CsA and SFA binding

Sedrani et al. recently published the X-ray structure of cyclophilin A complexed with a macrolide analogue of the cyclic region of SFA [32] which binds in the active site cleft of the cyclophilin, as does CsA [33]. Fig. 3 shows a molecular graphics comparison of CsA and the SFA macrolide analogue bound in the active site of cyclophilin A. The theoretical contact points between the cyclophilin and the drug were calculated and are indicated by rings. It is interesting to see that although they both bind into the active site, CsA has nine contact points to SFA eight, which is surprising given its greater affinity for the cyclophilin. The active site appears to have a slightly different conformation between the two bound forms, which may account for the two drugs sharing only six contact points, but more importantly the two interactions CsA has with the tryptophan residue in the xLD motif (Trp121) are not shared with the macrolide analogue of the cyclic region of SFA. Why therefore does this residue correlate with the cyclophilins sensitivity to SFA? It could possibly be due to conformational changes induced by the presence of phenylalanine or histidine within the active site itself that lead to its reduced affinity. What is missing from this picture is the location of the linear region of SFA that is attached to the cyclic ring of the macrolide. From the position of the macrolide component residues in the structure, the linear 'tail' region will extend down from the active site taking it past the tryptophan residue. This could therefore contact this residue and lead to the observed effect changing this residue has on SFA sensitivity.

3.4. Tryptophan fluorescence titrations

To confirm that SFA does in fact bind near to, and potentially contact, the tryptophan residue in the xLD motif by virtue of its 'tail' region, fluorescence assays were performed as previously reported by Liu et al. [17] in the analysis of CsA binding to various cyclophilin mutants. The proximity of the drug to the tryptophan residue causes an increase in its resonance through changes in its local environment which lead to an increase in its emission intensity when excited. By monitoring the emissions in the presence of an increasing drug concentration allows us to see if the drug binds near the residue.

Fig. 4 shows the plots of the change in tryptophan fluorescence seen upon drug addition to SpCyp1–5 and the SpCyp3-F128W mutant. It is evident that no change in fluorescence is seen with SpCyp3 and SpCyp5 upon the addition of either drug, which is as expected as they possess no tryptophan in their xLD motif. However, with the addition of either CsA or SFA, SpCyp1, SpCyp3-F128W and SpCyp4 show an increase in fluorescence to a maximum that varies between the cyclophilins. This variation is due to the bound drug having differing effects on tryptophan fluorescence when saturating the different cyclophilins, which may be due to differences in the distance and geometry of the bound drug to the tryptophan or

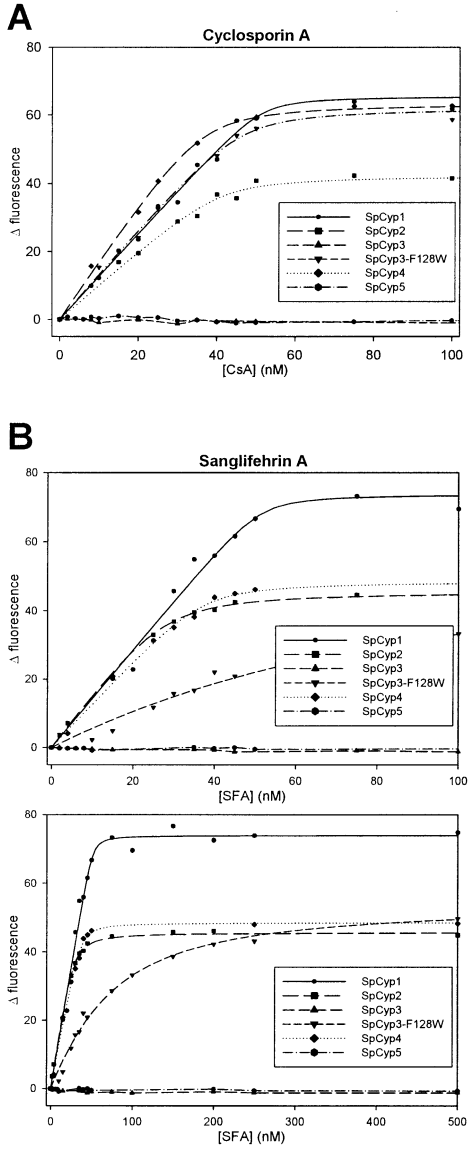

Fig. 4. Fluorescence titration graphs of the purified SpCyps. (A) shows the change in fluorescence upon the addition of CsA over the range of 0–100 nM and (B) shows the change in fluorescence upon the addition of SFA over the range of either 0–100 pM (upper) or 0–500 pM (lower).

in the level of solvation change around the tryptophan upon drug binding.

These data confirm that some part of SFA does bind in close proximity to the tryptophan residue in the xLD motif. This is most likely the 'tail' region of the drug given the position and orientation of the cyclic region in the active site.

4. Discussion

We have reported here the cloning, expression and catalytic characterisation of four SpCyps, that all have identified orthologues in humans [12]. We had previously reported the cloning and characterisation of SpCyp3 [12], which was also investigated in this paper.

The SpCyps have shown comparable activity on the tetrapeptide substrate succ-AAPF-pNA to those orthologues that have previously been reported from other species. SpCyp2 and SpCyp3 showed the greatest activity which was roughly five times that of the lowest, SpCyp1.

CsA sensitivity assays showed SpCyp1, SpCyp2 and SpCyp4 to have comparable K_i's but SpCyp3 and SpCyp5 showed a lower sensitivity to the drug. This reduced sensitivity can be correlated to a specific tryptophan residue in the xLD motif, as has been previously reported by Liu et al. using mutagenesis experiments with human cyclophilin A [17] and Hoffmann et al. with human cyclophilin 40 [31]. The presence of a phenylalanine conferred an order of magnitude lower sensitivity than tryptophan, with a histidine conferring an intermediate sensitivity.

Inhibition assays with the novel cyclophilin-targeting immunosuppressant drug SFA confirmed that the drug is more potent than CsA at inhibiting the SpCyps, as previously reported for human cyclophilin A [6]. The results also showed that the sensitivity of the cyclophilins to SFA can be correlated to the same specific tryptophan residue as CsA.

Construction of a cyclophilin 3-F128W mutant confirmed directly that the sensitivity of cyclophilin 3 to both CsA and SFA can be correlated to the 'x' residue in the xLD motif, and given the results of the wild-type proteins, this can be inferred to be true for all cyclophilins. This observation implies that part of the SFA molecule binds in close proximity to the 'x' residue. The comparable catalytic activity of cyclophilin 3 and the F128W mutant agreed with Liu et al.'s observation that variations in this position do not affect the prolyl isomerase activity of the cyclophilin [17].

Molecular graphics analysis of CsA bound to cyclophilin A [33] compared to the recent structure of a macrolide analogue of the cyclic region of SFA bound to cyclophilin A [32] showed that the cyclic region does not bind in the proximity of the tryptophan residue. However, the binding of SFA in close proximity to the tryptophan residue in the xLD motif has been confirmed by tryptophan fluorescence titrations that show an increase in fluorescence in the presence of the drug. This leads us to the conclusion that it is the linear 'tail' region of SFA that binds in close proximity to the variable 'x' residue in the xLD motif and this therefore leads to the observed variability in sensitivity of the cyclophilins to SFA.

SpCyp1 and SpCyp2 show similar maxima in the fluorescence titrations with CsA and SFA, but SpCyp3-F128W and SpCyp4 have lower maxima with SFA than with CsA, suggesting that SFA binds further away from, or in a different geometry in relation to, the tryptophan than CsA in these two cases. The SpCyp3-F128W mutant also showed a significantly lower affinity for SFA than CsA in the fluorescence titrations as a greater concentration of SFA is required for it to reach its fluorescence maximum. This reduced affinity is not reflected in the inhibition constant, inferring that this reflects a structural feature that significantly affects SFA, but not CsA, binding in the vicinity of the tryptophan and that this doesn't affect its overall binding. We would conclude that this feature affects the positioning of the 'tail' region about the tryptophan, which therefore appears to have little effect on the drugs affinity, rather than in the binding of the cyclic region. This is supported by Sedrani et al., who reported that the macrolide analogue of SFA's cyclic region bound to human cyclophilin A with an almost identical affinity to that of the complete SFA molecule [32].

The reasons why reduced drug sensitivity is seen with those cyclophilins possessing a phenylalanine or histidine in place of this tryptophan cannot be explained by these fluorescence data. As the positioning of the 'tail' around the tryptophan appears to have little effect on SFA's affinity for the cyclophilin, its repositioning in the presence of the other two residues is unlikely to cause the significant change in affinity we have observed. This therefore makes changes in the structure of the active site that occur in the presence of the other two residues the most likely cause.

Further work is therefore needed to identify all the factors that affect SFA binding, with the SpCyps representing a good model group for this to be performed with. The effect of the macrolide analogue of SFA's cyclic region on the catalytic activity of these cyclophilins will show whether or not it is structural changes within the active site that lead to the reduced affinity in the phenylalanine and histidine containing cyclophilins. Should its inhibition constants be those seen with the complete SFA molecule then this would infer that it is, whereas inhibition constants that show an affinity about those of the tryptophan containing cyclophilins would infer that this change in affinity is due to an interaction of the 'tail' region. Structural data on a cyclophilin with the complete SFA molecule will be of great use as it will allow us to better understand the positioning of the 'tail' region in relation to the tryptophan residue, and if also possible with a cyclophilin possessing a phenylalanine or histidine, a comparison might allow us to identify the factors that lead to reduced drug affinity.

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