Biochemical Features of Recombinant Human Cyclophilin J

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Abstract. *Aim: To characterize the biochemical features of the newest member of cyclophilin family of peptidyl-prolyl cis/trans-isomerases (PPIases), cyclophilin J (CYPJ). Materials and Methods: PPIase assays were performed on purified hCYPJ and its mutated variants. The substrate specificity, half-maximal inhibitory concentration (IC_{50}) of cyclosporin A (CsA) inhibition and circular dichroism (CD) spectrum of CYPJ were measured. Mercury pathway profiling luciferase assays were also performed. Results: The catalytic number/Michaelis constant (k_{cat}/K_M) value of CYPJ was 9.5×10^4 $s^{-1}M^{-1}$. CYPJ additionally catalyzed norleucine-proline, isoleucine-proline and glutamine-proline peptides compared to CYPA and Escherichia coli PPIases. CYPJ was inhibited by CsA in a dose-dependent manner with IC_{50} of 12.1±0.9 μM. The CD spectrum of CYPJ was similar to CYPA. CYPJ significantly up-regulated the transcription of E-box, E2F, retinoblastoma (Rb), p53, activator protein 1 (AP1), NF-κB and phospho-cAMP response element (CRE) cis-response element in 293T cells. Conclusion: CYPJ structurally resembles CYPA. It is sensitive to inhibition by CsA and plays a role in regulating cell growth, proliferation, and apoptosis.*

Cyclophilins (CYPs) are a large family of peptidyl-prolyl *cis/trans*-isomerases (PPIases, EC 5.2.1.8) that can selectively bind to cyclosporin A (CsA). As an immunosuppressive drug, CsA interrupts the T-cell signalling pathway by inducing false folding of T-cell membrane protein after forming a CsA–cyclophilin complex (1). CYPs are involved in a large variety of biological events across cells, including immunosuppression, signal transduction, the allosteric effect and assembly of proteins (2-4), mitochondria-related apoptosis (5), RNA splicing (6, 7), mitosis (8, 9), tumourigenesis (10, 11) and HIV infection (12-14).

CYPs are highly conserved proteins that exist extensively and have been isolated from a large variety of organisms including Bacillus, *Escherichia coli*, yeast, plants and eukaryotes (15, 16). To date, 17 different types of CYPs have been identified in the human genome, with molecular weights between 8 and 150 kDa. The coding sequence of the latest member of human cyclophilin family, CYPJ, was first reported as one of the two transcript variants of human peptidylprolyl isomerase-like 3 (*PPIL3B*) gene in 2001 (17). Human CYPJ protein was purified and crystalized by Huang *et al*. in 2004, and showed 72% sequence identity to CYP10 in *Caenorhabditis elegans* and 50% to human CYPA (18). hCYPJ was reported to bind CsA with similar active-site structure but with somewhat different binding details from those for hCYPA–CsA complex (18).

CYPJ contains the PPI consensus sequence [F/Y]–X(2)–[S/T/C/N/L/V]–X–F–H–[R/H]–[L/I/V/M/N]–[L/I/V/M]–X(2)–F–[L/I/V/M]–X–Q–[A/G]–G (19), suggesting its role as a PPIase. However, the biochemical properties of CYPJ as a PPIase remain to be explored. Herein, we studied the enzymatic kinetics of CYPJ and its interaction with binding partner CsA.

Materials and Methods

Protein expression and purification. Construction of CYPJ expression plasmid and protein expression was performed as previously described (18) except that *E. coli* Rosetta cells (Novagen, Shanghai, China) were used for protein expression in this study. Purified protein

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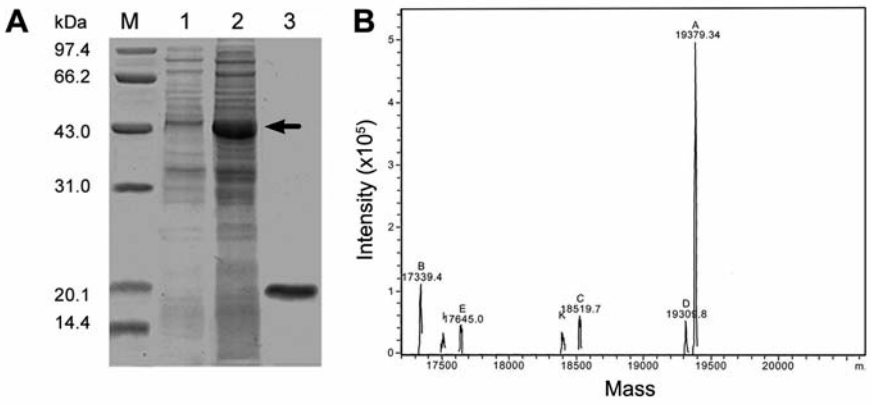


Figure 1. *Overexpression and purification of cyclophilin J (CYPJ). A: SDS-polyacrylamide gel electrophoresis of uninduced (lane 1) and IPTG (300 μmol/l)-induced (lane 2) protein expression of recombinant CYPJ, purified digested CYPJ protein (lane 3). Arrow indicates the recombinant CYPJ band. B: LC/MS graph showing the molecular weight of purified CYPJ.*

was analyzed by liquid chromatography/mass spectrum (LC/MS) (20), or kept in PPIase buffer (50 mM HEPES, pH 8.0, and 86 mM NaCl) at 4˚C until use. Samples were separated by 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and stained with coomassie brilliant blue (Sigma-Aldrich, Shanghai, China).

Biochemical assays. PPIase activity assays of CYPJ were performed at 10˚C in quartz cuvettes with a path length of 1 cm under vigorous stirring with a U-3000 spectrophotometer (Hitachi, Tokyo, Japan) in 35 mM HEPES buffer at pH 7.8. Enzyme activities of 100 nM CYPJ towards the substrate Suc-A-A-P-F-pNA (Sigma-Aldrich, Shanghai, China) were measured using the protease-free assay according to Janowski *et al*. (21) in the additional presence of isomerase-specific protease chymotrypsin (0.83 mg/ml final concentration).

CsA inhibition assays were carried out as described above using 105.5 nM CYPJ and the samples were pre-incubated for 60 min on ice with differing amounts of CsA (Sigma-Aldrich, Shanghai, China) in 50% (v/v) ethanol/water.

For the substrate specificity assay, Suc-A-Xaa-P-F-pNA (Xaa represents a serial of amino acid residuals) was used as substrate, and the catalytic constant catalytic constant/Michaelis constant (kcat/KM) values of CYPJ, CYPA, *E. coli* PPIase a and *E. coli* PPIase b were tested and calculated.

Circular dichroism (CD) spectroscopy. CD spectroscopic measurements of CYPJ and CYPA were performed on a CD spectrometer (Jasco, Shanghai, China) at 10˚C at a wavelength of 200-250 nm. CYPA concentration was 10 μM in 10 mM phosphate buffer, pH 7.5, d=0.1 cm. CYPJ concentration was 10 μM in 50 mM phosphate buffer, pH 8.5, d=0.1 cm. Each curve represented a combined result of five scans. For the thermal stability CD spectroscopic analysis, the CYPJ solution in the cuvette was covered with paraffin oil to prevent evaporation. CYPJ was incubated for 10 min at 25 ˚C and a 10 ˚C increment between 40-90˚C before the CD spectrum was measured.

Fluorescence spectroscopy. Peptide substrate was mixed with 3.5 μM CYPJ. The fluorescence emission curve of CYPJ was obtained using a FP-777 spectrofluorometer (Jasco, Shanghai, China) by subtracting the fluorescence intensity of respective peptide substrate from that of the whole reaction mixture, and then corrected for the internal standard filtering effect (22).

Luciferase assay. The Mercury™ pathway profiling luciferase system (Clontech, CA, USA) was used for testing the effect of CYPJ on multiple signalling pathways according to manufacturer's instructions. pCMV-CYPJ-myc plasmid was constructed by cloning the coding sequence of human *CYPJ* into pCMV-myc vector. 293T cells (ATCC, Manassas, USA) (3×10^4) were inoculated in 24-well plates and cultured for 18-24 h at 37˚C in an atmosphere of 5% CO_2. Reporter plasmid (100 ng of pAP1-Luc, pCRE-Luc, pNFκB-Luc, pE2F-TA-Luc, pMyc-TA-Luc, pp53-TA-Luc or pRb-TA-Luc), 10 ng inner referencing plasmid pRL-SV40 and 400 ng either pCMV-CYPJ-myc (+ve) or pCMV-myc (–ve) vector were simultaneously transfected with Lipofectin Reagent (Invitrogen, Shanghai, China). Cells were lysed after 5 days' culture, and the luciferase activity was detected using an LB9507 luminometer (Berthold Technologies GmbH & Co. KG, Bad Wildbad, Germany). The luminance intensity was recorded. For each signalling pathway, the fold increase of transcription level was calculated as =M2+M1+/M2−. (where M2: mean luminance from the reporter plasmid; M1: mean luminance from the inner referencing plasmid; +/–: +ve or –ve group.

Statistical analysis. Data are presented as the mean values and standard deviation of the sample. Statistical analysis was performed using the two-tailed Student's *t*-test. A value of $p<0.05$ was considered statistically significant.

Results

Expression and purification of recombinant human CYPJ protein. In order to characterize CYPJ biochemically, we expressed and purified recombinant CYPJ from pTXB1 vector in *E. coli*. After affinity chromatography and gel chromatography purifications, high-purity recombinant CYPJ

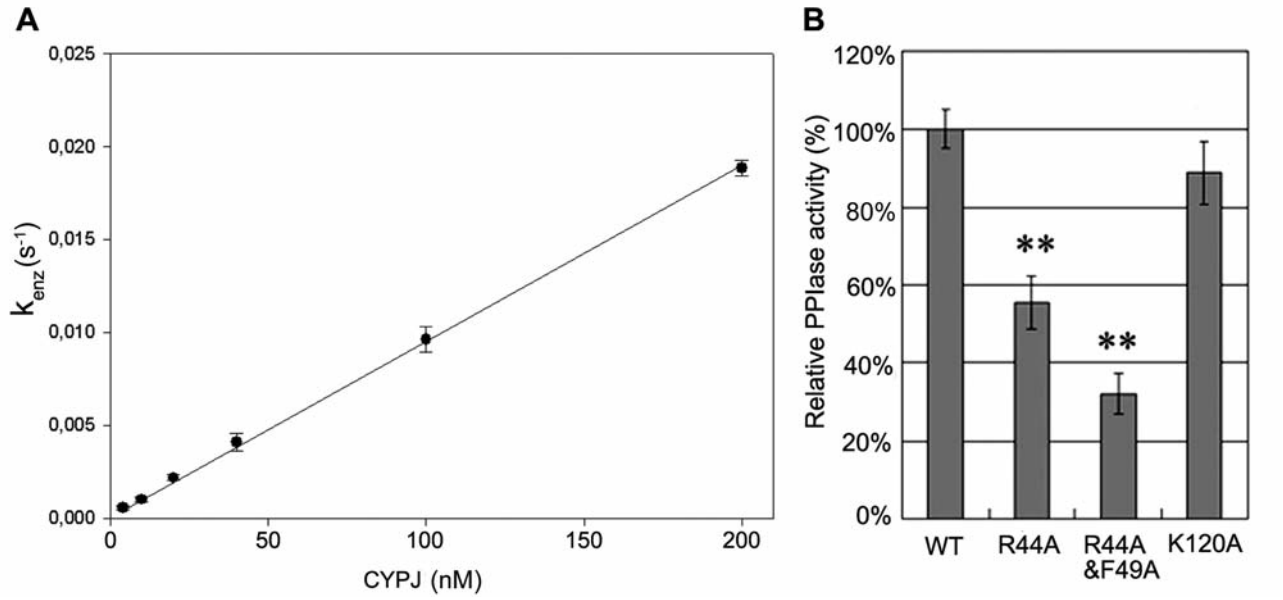


Figure 2. *Peptidyl-prolyl cis/trans-isomerase (PPIase) activities of cyclophilin J (CYPJ). A: Determination of catalytic number/Michaelis constant (k_{cat}/K_M) for CYPJ using the substrate Suc-A-A-P-F-pNA. B: Effect of site mutations on the PPIase activity of CYPJ. **p<0.01. WT=100±5.2%, $CYPJ^{R44A}$=57±7.3%,$CYPJ^{R44A\&F49A}$=32±6.0% and CYPJK120A=87±8.5%, n=5.*

Table I. *Substrate specificity of cyclophilin J (CYPJ) (s^{-1} M^{-1}) in comparison to other cyclophilins as relative catalytic number/ Michaelis constant (k_{cat}/K_M) values. The value for Suc-A-A-P-F-pNA (k_{cat}/K_M= 9.5×10^4 s^{-1} M^{-1}) was arbitrarily set to 100%.*

Substrate amino acids	CYPJ	hCYPA	*E. coli* PPIase a (29)	*E.coli* PPIase b (30)
Alanine	100	100	100	100
Norleucine	90	0	0	0
Valine	85	100	0	0
Isoleucine	60	0	0	0
Leucine	38	85	35	48
Glycine	19	37	27	38
Lysine	13	29	5	8
Glutamine	31	0	0	0
Glutamate	32	67	25	16
Phenylalanine	11	44	29	25
Histidine	6	19	16	7
Tryptophan	5	0	0	0

E. coli PPIase: *E. coli* peptidyl-prolyl *cis/trans*-isomerase.

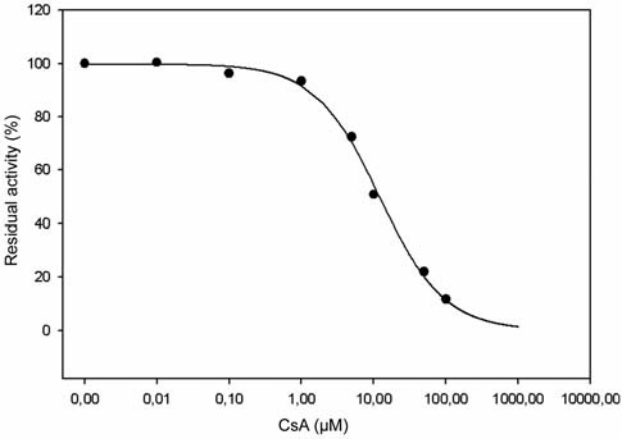


Figure 3. *Cyclosporin A (CsA) inhibition of recombinant cyclophilin J.*

protein was obtained. SDS-PAGE showed an overexpression of recombinant protein of approximately 44 kDa (Figure 1A) and a single band of purified digested protein of approximately 19 kDa (Figure 1B). The relative molecular weight of the purified protein was 19379.34 Da, as determined by LC/MS (Figure 1C).

PPIase activity of CYPJ. The PPIase enzymatic activity of hCYPJ was determined. In the chymotrypsin-coupled assay with different concentrations of the peptide substrate, the recombinant human CYPJ appeared to follow Michaelis–Menten kinetics. The values of k_{cat} and K_M were determined by a double reciprocal Lineweaver–Burke plot of 1/v against 1/[S], yielding a k_{cat}/K_M value of 9.5×10^4 $s^{-1}M^{-1}$ (Figure 2A). Several key amino acids (R44, F49, K120) have previously been reported to be important for the catalytic activity of CYPA protein (23). The corresponding mutants

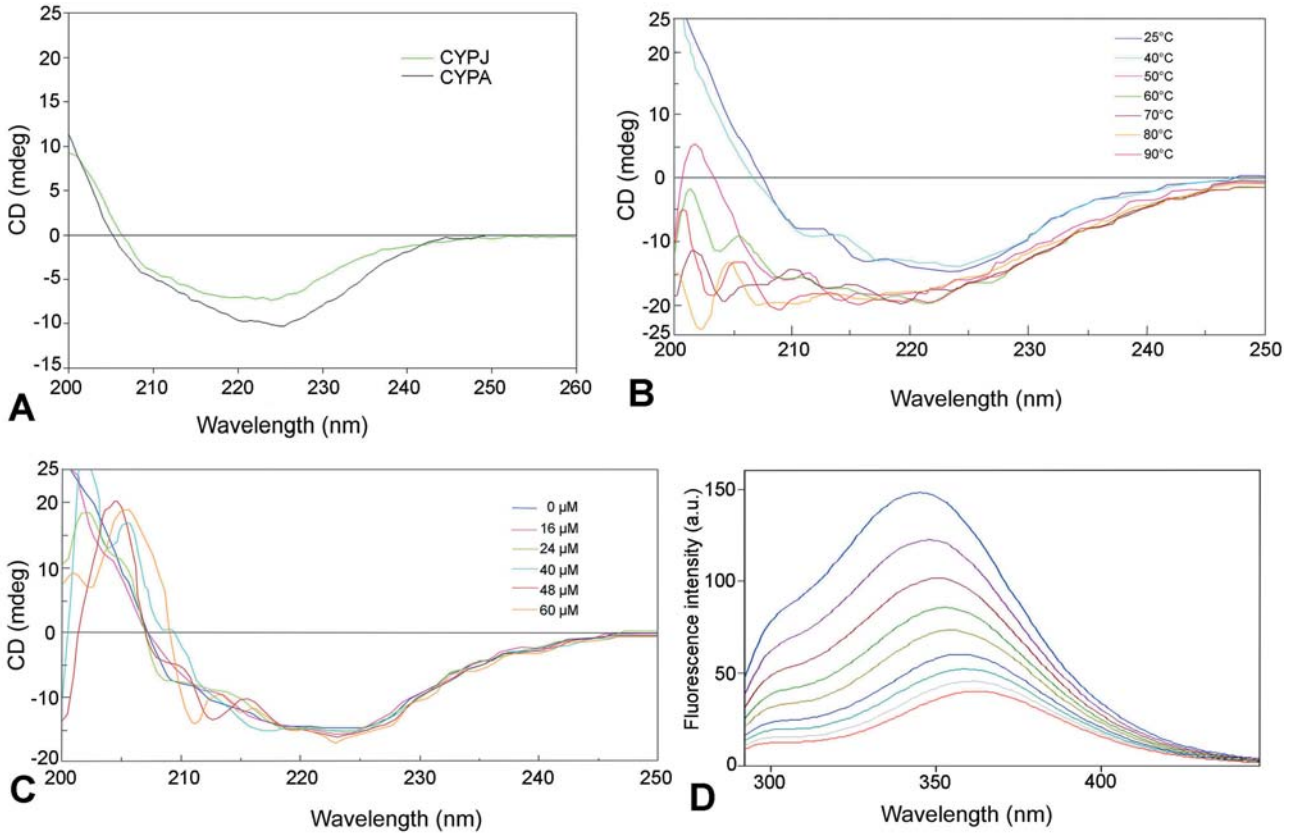


Figure 4. *Circular dichroism (CD) and fluorescence spectrum of cyclophilin J (CYPJ). A: CD spectrum of CYPJ in comparison to CYPA at 10˚C. B: Thermal stability CD spectrum of CYPJ. C: Effects of cyclosporin A (CsA) concentration on the CD spectrum of CYPJ. D: Fluorescence spectrum of CYPJ with different concentrations of peptide substrate. Lines from top to bottom represent using 0, 0.66, 1.32, 1.98, 2.64, 3.3, 3.96, 4.62, 5.28 μM peptide substrate respectively.*

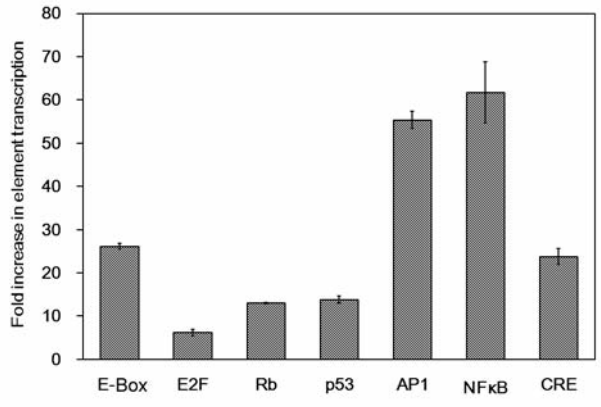
were generated (R44A, R44A&F49A, and K120A) for CYPJ and their residual PPIase activities were assayed and compared to those of the wild-type CYPJ protein. The R44A, R44A&F49A, and K120A CYPJ mutants displayed 57%, 32% and 87% of the wild-type enzyme activity, respectively, that was consistent with the roles of these residues in catalysis (Figure 2B).

To further characterize the PPIase enzyme activity of CYPJ, different Suc-A-Xaa-P-F-pNA were used as substrates in PPIase assay and the k_{cat}/K_M values of CYPJ were compared with those of hCYPA, *E. coli* PPIase a and *E. coli* PPIase b (Table I). The results show that in addition to the common substrates for CYPA and *E. coli* PPIases, CYPJ also catalyzes the *cis/trans* isomerization of norleucine-proline, isoleucine-proline and glutamine-proline peptides.

The inhibitory effect of CsA on PPIase activity of CYPJ was also examined. The results showed that CYPJ was inhibited by CsA in a dose-dependent manner and the IC_{50} was determined to be 12.1±0.9 μM (Figure 3).

Physicochemical properties of CYPJ. CD spectroscopic analysis showed that recombinant CYPJ comprised of 10.5% α-helix, 51.6% β-sheets and 37.9% coil. The CD spectrum of CYPJ resembled that of CYPA (Figure 4A). During thermal denaturation, the secondary structure of CYPJ remained stable below 40˚C. When reaching 50˚C, the number of secondary structures significantly decreased in CYPJ with the appearance of many random conformations (Figure 4B). We also tested the CD spectrum of CYPJ with different concentrations of CsA. With increasing CsA concentration, the ratio of α-helix increased, while the number of coils decreased (Figure 4C).

The excitation spectrum of CPYJ revealed 280 nm to be its maximum fluorescence excitation wavelength. With increaseing concentration of peptide substrate, the fluorescence intensity of CYPJ decreased, with redshifted maximum emission wavelength (Figure 4D), indicating an interaction of the protein with substrate. The induction by substrate caused changes in the conformation of tryptophan.



Cis-response Element	Transcription factor	Singnaling pathway
E-Box DNA	MYC/MAX	Cell proliferation
E2F DNA	E2F/DP1	Cell cycle progression
Rb response element	Rb	Cell cycle progression
p53 response element	p53	Cell growth/apoptosis
Activator protein 1 (AP1)	c-JUN/c-FOS	JNK pathway
Nuclear factor κB (NFκB)	NFκB	NFκB pathway
CAMP response element (CRE)	ATF2/CREB	JNK/p38&PKA pathway

Figure 5. *Effect of cyclophilin J (CYPJ) on multiple signaling pathways. The chart shows the fold increase in transcription of the reporter genes in Luciferase with induction by CYPJ. Error bars indicate the standard error (n=3).*

Involvement of CYPJ in signaling pathways. To investigate the involvement of CYPJ in a number of signaling pathways, we performed luciferase assays in 293T cells transfected with a number of different reporter plasmids (Figure 5) together with inner referencing vector pRL-SV40 and CYPJ expression vector pCMV-CYPJ-myc. pCMV-myc empty vector was used instead of pCMV-CYPJ-myc as a negative control. Results showed that compared to the empty vector, the pCMV-myc plasmid expressing CYPJ significantly increased the transcription of luciferase reporter gene with the E-box ($p=2.251\times10^{-6}$), E2F ($p=0.00341$), Rb ($p=1.144\times10^{-4}$), p53 ($p=1.166\times10^{-5}$), AP1 ($p=4.144\times10^{-6}$), NFκB ($p=9.802\times10^{-4}$) or CRE ($p=1.831\times10^{-4}$) cis-response element in 293T cells (Figure 5), suggesting strong up-regulatory effects of CYPJ in the corresponding signaling pathways.

Discussion

In this study, we expressed and purified the new member of human cyclophilin family, CYPJ, and investigated its biochemical features. We demonstrated that CYPJ is a PPIase that is sensitive to inhibition by CsA.

The predicted that the molecular weight of human CYPJ was 18,154 Da, while LC/MS showed our recombinant CYPJ to be 19,379.34 Da, most likely due to the MASSRVDG and LEGSS residue on the *N* and *C* ends of CYPJ after cleavage from the pTXB1-expressed fusion protein. Therefore, discrepancy might exist between the properties of our recombinant protein and its natural counterpart.

Compared to the k_{cat}/K_M value of 1.4×10^7 $s^{-1}M^{-1}$ for CYPA (24), CYPJ has a k_{cat}/K_M of 9.5×10^4 $s^{-1}M^{-1}$, which is 147-fold lower. This could be due to the difference in the molecular structure between CYPA and CYPJ. Structural difference results in the varied PPIase activity among the cyclophilin family, *e.g.* there is a 432-fold difference between the PPIase activity of CYP6 and CYP10 in *C. elegans* (25). Similarly, the IC_{50} for CsA against CYPJ was 12 μM compared to 19 nM for CYPA, that could be explained by the difference in CsA-CYP binding site. Our CD spectroscopy revealed resemblance in the secondary structure of CYPA and CYPJ, that was in accordance with the crystal structure of CYPJ (18).

The biological function of CYPJ (or PPIL3b) remains largely a mystery. *PPIL3* mRNA was ubiquitously transcribed in adult human tissues, poorly expressed in human brain tissues and overexpressed in human glioma tissues (17, 26). *PPIL3B* was reported to be down-regulated in anandamide-induced apoptosis of human neuroblastoma SH-SY5Y cells (27). PPIL3 was also discovered as a direct binding partner of apoptin, which held apoptin in the cytoplasm of tumour cells, preventing the migration of apoptin into cell nucleus and killing of the tumor cell (28). Our data identified that CYPJ promoted the transcription of cis-response elements of several pathways related to cell growth, proliferation, and apoptosis (Figure 5). Our preliminary data also showed up-regulation of CYPJ expression in human hepatocellular carcinoma (Chen J, unpublished data). Overall, these findings suggested a strong involvement of CYPJ in carcinogenesis.

Conclusion

CYPJ structurally resembles CYPA. It has PPIase activity that is sensitive to inhibition by CsA and might play a role in regulating cell growth, proliferation, and apoptosis. This study adds to our current knowledge of cyclophilin family of proteins and paves the way for future research on the biological function of CYPJ.

Acknowledgements

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