Structural and molecular characterization of human FK506-binding protein 25 (FKBP25), a nuclear immunophilin

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**STRUCTURAL AND MOLECULAR
CHARACTERIZATION OF HUMAN FK506-BINDING
PROTEIN 25 (FKBP25), A NUCLEAR IMMUNOPHILIN**

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2016

**Structural and molecular characterization of human
FK506-binding protein 25 (FKBP25), a nuclear
immunophilin**

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School of Biological Sciences

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This thesis is dedicated to my beloved parents.

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Abstract

Human FK506 binding protein (FKBP25), a 25 kDa nuclear protein, is a member of the FK506-binding protein family with peptidyl-prolyl *cis-trans* isomerase (PPIase) activity. It has a conserved C-terminal FK506 binding domain (FKBD), which binds with immunosuppressive drugs such as FK506 and rapamycin and a unique N-terminal helix-loop-helix domain (HLH). These two domains are linked through a long flexible loop. FKBP25 is known to interact with proteins like Nucleolin, MDM2, YY1 and most importantly HDAC1/2. While the structures of two the individual domains of human FKBP25 are known, the structure of full-length FKBP25 and the molecular mechanism of its interaction with nucleic acids remain unknown. The main objective of this thesis research is to perform the structural and molecular characterization of FKBP25 in order to explore the mechanism of its interaction with other proteins, nucleic acids and drugs with an aim of delineating its molecular function.

In this study, we determined the crystal structure of human FKBD25 in complex with FK506 drug and attempted to explore the mechanism by which FKBP25 shows differential binding affinity to FK506 and rapamycin (200-fold higher affinity), which is unique feature of FKBP25 among other FKBP. Later we also determined the nuclear magnetic resonance (NMR) solution structure of the human full-length FKBP25. The topology of FKBP25 showed that the HLH and FKBD are connected by a long and unstructured flexible linker between the domains. The N-terminal domain consists of five α -helices (Helix-Loop-Helix domain), whereas the C-terminal domain shows a canonical FKBD fold which consists of six antiparallel β -strands and a short central α -helix. Further using gel shift assay, we showed that FKBP25 can interact with DNA in sequence-independent manner. This binding was confirmed by biophysical assays including isothermal titration calorimetry (ITC), tryptophan fluorescent quenching and NMR experiments. The binding affinity was estimated around 1.23 μ M. We then identified the DNA binding site on FKBP25 by NMR titration and confirmed that mutations of some of the amino acids from the DNA binding site caused reduced DNA binding.

We also observed intermolecular NOEs between FKBP25 and DNA. Based on multinational studies and NMR data, we performed docking of FKBP25 with DNA. The FKBP25-DNA complex model revealed that both N-terminal domain and the basic loop of the C-terminal domain are important for nucleic acid recognition. Sequence alignment of FKBP25 with other human FKBP and homologs of FKBP showed that the basic loop is exclusively present in human FKBP25 and could be important for nucleic acid binding. The fourth helix of the HLH domain forms major-groove interactions and the basic FKBD loop cooperates to form interactions with an adjacent minor-groove of DNA. The FKBP25-DNA complex model provides structural and mechanistic insights into the nuclear immunophilin-mediated nucleic acid recognition.

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Abbreviations

1D	One-dimensional
Å	Angstrom
Abs	absorbance
aa	Amino acid
bp	Base pair
BSA	Buried surface area
CaM	Calmodulin
CaN	Calcineurin (protein phosphatase 2B, PP2B)
COSY	Correlated spectroscopy
CsA	Cyclosporin A
CK2	Casein Kinase II
CSP	Chemical shift perturbation
Cyp	Cyclophilin
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide phosphate
EDTA	Ethylene-diamine-tetraacetic acid
FKBP	FK506-binding protein
FKBD25	FK506 binding domain (109-244 aa)
hFKBP25	Human FK506-binding protein 25
HDAC	Histone deacetylase
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
Hsp90	Heat shock protein 90
HSQC	Heteronuclear single quantum correlation
IPTG	Isopropyl-β-D-thiogalactopyranoside
MD	Molecular dynamics
MDM2	Mouse double minute 2
mTOR	mammalian target of rapamycin

NFAT	Nuclear factor of activated T-cell
Ni-NTA	Nickel-nitriloacetic acid
NMR	Nuclear magnetic resonance
NOE	Nuclear Overhauser enhancement
NOESY	Nuclear Overhauser enhancement spectroscopy
OD	Optical density, absorbance
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PDB	Protein data bank
PEG	Polyethyleneglycol
PPIase	Prolyl <i>cis/trans</i> isomerase
ppm	Parts per million
rpm	Rotations per minute
SDS	Sodium dodecylsulfate
SDS-PAGE	Sodium dodecylsulfate - polyacrylamide gel electrophoresis
SUMO	Small Ubiquitin-like Modifier
TAE	TRIS-acetate-EDTA
TOCSY	Total correlated spectroscopy
TPR	Tetratricopeptide repeat
TRIS	Tris-(hydroxymethyl)-aminomethane
UV	Ultra violet

Chapter 1

Introduction

1 Introduction

Proteins play a fundamental role in virtually every biological process. In the ribosome, all proteins are synthesized as linear polypeptide chains and to become biologically active, they fold into a unique three-dimensional structure. Because of malfunction of the protein folding machinery in biological systems, proteins may fail to fold correctly and efficiently which may lead to several diseases such as cystic fibrosis, Alzheimer's disease and Parkinson's disease (Chaudhuri and Paul, 2006; Cohen and Kelly, 2003). Although the information for correct folding is encoded by the amino acid sequence for most proteins (Anfinsen, 1973), living organisms are additionally equipped with efficient folding machinery, consisting of chaperones (Bukau et al., 2006; Caplan et al., 2007), protein disulfide isomerases (Ellgaard and Ruddock, 2005) and peptide bond isomerases (Fischer and Aumuller, 2003). The *cis* / *trans* interconversion of peptide bonds is catalyzed by peptide bond isomerases which play an important role in protein folding. Peptide bond possesses partial double bond character due to which it can adopt either *cis* or *trans* conformation. There are two classes of peptide bond isomerases (i) the secondary amide peptide bond isomerases (APIases) (Schiene-Fischer et al., 2002) and (ii) the major class of the peptidyl prolyl *cis-trans* isomerases (PPIases) (Fischer and Aumuller, 2003).

1.1 Peptidyl-prolyl *cis-trans* isomerases (PPIase)

Peptide bonds in a protein can be present in *cis* or *trans* conformation. *Trans* conformation of peptide bonds are favored over *cis* because of less steric hindrance and hence peptide bonds exist mostly in the *trans* form. Because of its unique side chain structure, the proline peptide bond is different from the peptide bond formed by other amino acids (proline is the only amino acid in which the amide nitrogen participates in a ring formation (See figure 1A). The Xaa-Pro peptide bond exists both in *cis* and *trans* conformation (*cis* can be 10-30%, depending on the nature of the Xaa-Pro bond). The *cis* and *trans* configurations of a Xaa-Pro peptide bond have a very small difference in their free energy. Because of those *cis/trans* isomerization requires relatively high activation energy, and thus *cis* / *trans* isomerization of Xaa-Pro peptide bond requires a family of enzymes called peptidyl-prolyl *cis-trans* isomerase (PPIase). The PPIase is a ubiquitous protein present in all forms of life.

Figure 1.1 *Cis* and *trans* conformation of prolyl peptide bond. (A) Structure of proline amino acid, showing N atom involved in a ring formation with its side chain atoms which is unique among all amino acids (B) The *cis* / *trans* interconversion of prolyl peptide bond is a slow process catalyzed by PPIase. Such isomerization is important for protein folding (Wang and Heitman, 2005).

There are 3 subfamilies within the class of PPIase and these subfamilies are unrelated to each other in their amino acid sequences, and substrate and inhibitor specificities. The three PPIase subfamily members are (i) FK506-binding proteins (FKBPs), (ii) cyclophilins (CyPs) and (iii) parvulins (Pin). The members of the first two families (FKBPs and CyPs) are also called immunophilins as they are shown to bind with some immunosuppressive drugs like FK506 (also known as tacrolimus) and cyclosporin A (CsA) which leads to immune suppression by the inhibition of T-cell proliferation. Although no sequence homologies exist between the three PPIases, the structure of the active site is very similar in all these enzymes, suggesting that the catalytic pathways utilized by FKBPs, cyclophilins and parvulins are closely related (Horowitz et al., 1994a). PPIases can consist of one or more PPIase domains and also other domains for protein-protein interaction and membrane anchoring. These additional segments have been found both at the N-terminal and C-terminal ends of the catalytic domain and may account for the regulation and specific localization of the enzymes (Galat, 2004). Catalysis of peptidyl prolyl *cis/trans* isomerization is not the only function of PPIases, as PPIases have been found to be associated with several other biological functions like native state isomerization (Andreotti, 2003), signal transduction, immunosuppression, gene regulation, DNA replication, cell cycle regulation, spermatogenesis (Crackower et al., 2003), Ca²⁺ homeostasis (Wehrens et al., 2004) and pathways like Bcl-2-dependent apoptotic pathways (Edlich et al., 2005; Galat, 2004) and p53 signaling pathway. These proteins are also associated with a number

of disease conditions like Parkinson's disease, Alzheimer's disease, malaria, cancer etc. Mutations in FKBP genes are related to the occurrence of congenital diseases such as the Leber's congenital amaurosis (LCA) and Williams Beuren syndrome (WBS) (Meng et al., 1998).

1.2 FK506 binding proteins (FKBPs)

FK506 binding proteins (FKBPs) are a major class of PPIases known to bind with immunosuppressant drugs like FK506 and rapamycin (for details see section 1.2.3) and FK506 binding proteins (also FKBPs) are conserved from *Archaea* to primates [reviewed by (Kang et al., 2008)]. In humans, a total of 16 different FKBPs have been reported (Figure 1.2). Members of this enzyme family can be found in all human tissues but are predominantly present in the nervous tissue. Most of the FKBPs are cytoplasmic proteins except FKBP25 which has been reported as a nuclear FKBP (discussed in detail in section 1.3).

FKBP12 is the simplest and most studied FKBP and like other FKBPs, it also serves as a molecular receptor of FK506 and rapamycin. FKBP12 stabilizes the calcium release channel by interacting with the ryanodine receptor (RyR) (Brillantes et al., 1994). FKBP12 is an inhibitor of the TGF β family receptor (TGF β R1) (Wang et al., 1996). It was demonstrated that another member of this group, FKBP51 is a positive regulator of androgen-dependent prostate cancer cell growth (Periyasamy et al., 2010). FKBP38 is a non-canonical FKBP as it does not bind to FK506. FKBP38 translocates the anti-apoptotic protein Bcl-2 to the mitochondrial outer membrane and protects cells from apoptosis (Wishart et al., 1994a). FKBP51 and FKBP52 are involved in nuclear localization of the glucocorticoid receptor (GR) in neurons.

binding. FKBP25 contains an N-terminal domain which helps in the interaction with histone deacetylase (HDAC) and YY1 (Yang et al., 2001).

1.2.2 Structure of FKBP

Several three-dimensional structures of this protein and its complexes with FK506, rapamycin, and other low-molecular-weight ligands have been solved by NMR and X-ray crystallography (Meadows et al., 1993; Michnick et al., 1991).

Human FKBP12 has been studied in detail to understand the FK506 interaction with the FK506 binding domain. This protein represents the minimal amino acid sequence displaying PPIase activity and FK506 binding and is therefore considered as the prototypic FKBP domain. It folds to a “half β -barrel” that consists of a five-stranded antiparallel β -sheet which wraps around a central α -helix and encloses the active site (Figure 1.3A).

The interior side of the β -sheets and the α -helix forms a hydrophobic cavity which accommodates immunosuppressive drugs like FK506 and rapamycin (Figure 1.3B and D). A total of fourteen residues (Tyr26, Phe36, Asp37, Arg42, Phe46, Glu54, Val55, Ile56, Trp59, Ala81, Tyr82, His87, Ile91 and Phe99) many of them highly hydrophobic, show direct contact (less than 4 Å) with the macrolide (FK506) (Figure 1.3C). Hydrogen bonds between FK506 and the residues Asp37, Glu54, Ile56, and Tyr82 provide additional stabilization to the drug/enzyme complex. In the FK506/FKBP12 complex, pipecolinyl moiety of FK506 rests on an aromatic cage formed by the side-chains of residues Tyr26, Phe46, Val55, Trp59 and Phe99 (Figure 1.3C and Figure 1.6). This cavity is also the binding site for the prolyl moieties of FKBP12 substrates (Figure 1.3D).

Figure 1.3: Three-dimensional structures of FKBP12 with or without FK506 showing Fk506 binding pocket (A) Crystal structure of free FKBP12 (PDB ID - 2PPN). All α -helices, β -strands, and loops are labeled and also colored in cyan, red and purple respectively. FKBD contains 5 β strands ($\beta 1$, $\beta 2$, $\beta 3$, $\beta 4$, and $\beta 5$) and one α helix (B) The structure of FKBP12–FK506 complex (PDB ID – 1FKJ) shown in same orientation and color code as free FKBP12. FK506 bound to FKBP12 in the FK506 binding pocket, is shown in yellow ball and stick representation. (C) The FK506-binding pocket of FKBP12 showing helices, beta strands and loops in same color code as used in (A) and (B). Five hydrophobic residues forming FK506 binding pocket are colored in yellow and labeled according to their positions in FKBP12. The four hydrophobic residues, Y26, F46, F99, W59 and Y82 which are important for FK506 binding, are located in $\beta 3$, $\beta 4$, $\beta 6$ strands, $\alpha 1$ helix, and 80s loop respectively. (D) The FK506 binding pocket of FKBP12 shown in the surface model using the same color code as free FKBP12. The shape of the cavity can accommodate five- and six-member rings as found in the structures of FK506.

1.2.3 Immunosuppression by FKBP

FK506 and rapamycin are well established immunosuppressive drugs, used in organ transplant action. These drugs bind to FKBP to mediate immunosuppression. Rapamycin (also known as sirolimus), is an immunosuppressant drug used to suppress the patient's immune system after an allogeneic organ transplant, especially kidney transplantation. In September 1999, FDA approved rapamycin as an immunosuppressive drug and this is being marketed by Pfizer under the trade name Rapamune. Rapamycin binds to FKBP and the complex of FKBP and rapamycin inhibits the protein kinase mTOR (mammalian target of rapamycin) (Sabers et al., 1995). This inhibition of mTOR, in turn, interferes with the activation of the protein kinase B / phosphatidylinositol 3-kinase (Akt / PI3K) signaling pathway (Fingar and Blenis, 2004), thus also inhibiting T-cell proliferation (Figure 1.4).

Figure 1.4: Mechanism of immunosuppression by rapamycin. Rapamycin binds to FKBP and FKBP/rapamycin complex binds to mTOR and prevents its activation. The inactivation of mTOR leads to inhibition of cell cycle progression and thus results into immunosuppression.

FK506, also called tacrolimus, is a 23-membered macrolide lactone and was discovered in 1984 (Figure 1.5). FK506 is used to prevent immune rejection during organ transplantation by reducing interleukin-2 (IL-2) production by T-cells and thus lower the risk of organ rejection. Other uses of FK506 include the skin condition vitiligo, treatment of atopic dermatitis (eczema) and severe refractory uveitis after bone marrow transplant. FK506 is sold under the trade names *Prograf*, *Advagraf*, and *Protopic*. The FK506 and rapamycin possess similar residues to bind FKBP's called as FKBP-binding domain. The residues which are dissimilar make effector domains and because of the different structure of effector domains, FK506 and rapamycin inhibits different protein and signaling pathway. FK506 binds to the FK506 binding domain of FKBP's to mediate immunosuppression. When T-cells get activated it increases the Ca^{++} level inside the cell. Ca^{++} binds to calmodulin which further activates a phosphatase called calcineurin. Activated calcineurin removes phosphate from phosphorylated NF-AT, a transcription factor. NF-AT can enter the nucleus to activate immune response genes like IL2, only when NF-AT is not phosphorylated. Dephosphorylation of NF-AT by calcineurin makes it active and helps in its nuclear transport. On treatment with FK506, FK506 makes a complex between FKBP and calcineurin and thus inhibits calcineurin to participate in NF-AT activation which finally prevents activation of T cells (Figure 1.6).

Figure 1.5: Structure of immunosuppressive drugs rapamycin and FK506. The FKBP-binding domains are colored in blue while effector domains are colored in pink and red for rapamycin and FK506 respectively. The Pipicolinyl moiety has been shown in green circle.

FK506 and rapamycin are known drugs for immunosuppression after organ transplantation. Pipecoliny moiety of these drugs is important for FKBP binding. It binds with the side chain of F99, Y26, F46 and W59 of FKBP to make an FKBP/drug complex.

Figure 1.6: The mechanism of immune suppression by FK506. FK506 makes a complex between FKBP and calcineurin which inhibits dephosphorylation of the transcription factor NF-AT. Phosphorylated NF-AT cannot enter into nucleus to activate transcription of immune response gene like IL-2 which eventually leads to immunosuppression (Steinbach et al., 2007)

1.2.4 FKBP as transcription regulators

Recent studies have established the role of FKBP in regulating gene transcription. FKBP12 interacts with YY1, a transcription factor, and relieves the repression activity of YY1 (detail about YY1 has been discussed in section 1.3.5). This interaction can be disrupted by FK506. FKBP25 also binds with YY1, but with the N-terminal domain and not its FK506 binding domain. FKBP25 also interacts with HDAC (discussed in detail in section 1.3.2). FKBP25 interacts with MDM2 and this interaction leads to increased auto-ubiquitination of MDM2. Due to a decrease in MDM2, p53 levels increase which in turn leads to increased p21 expression levels (Ochocka et al., 2009). Another example of transcriptional regulation by FKBP is HIF1 α target gene activation by FKBP38. Similar to FKBP25-MDM2 interaction, FKBP38 interacts with PHD2, an enzyme involved in prolyl-4-hydroxylation of HIF α . Hydroxylation of HIF1 α leads to its degradation. FKBP38 interaction with PHD2 leads to PHD2 ubiquitination and degradation which eventually results into an enhanced expression of HIF1 α target genes (Barth et al., 2009; Barth et al., 2007). FKBP52 modifies DNA binding property of IRF-4 (a known transcription factor), using its PPIase activity which suggests function of FKBP52 as a co-regulator for the transcriptional activity of IRF-4 (Mamane et al., 2000).

1.2.5 FKBP as histone chaperones

Histone chaperone (HC) is a histone-binding protein that regulates assembly and disassembly of the nucleosome *in vitro* or *in vivo*. Several HCs have been identified so far and a few of them are NAP1, CAF-1, HIRA, JDP2 and CIA / ASF1 (reviewed by (Eitoku et al., 2008)). Two yeast homologs of FKBP25, Fpr3p, and Fpr4p, also serve as histone chaperones. Both Fpr3 and Fpr4 contain an N-terminal domain with one basic and two acidic and a C-terminal PPIase domain (Hochwagen et al., 2005). The N-terminal highly basic and acidic regions of these nuclear FKBP interact with DNA and histone respectively. Fpr3 recognizes NLSs of histone H2B and helps to maintain recombinant checkpoint activity by preventing premature adaptation to DNA damage. The PPIase domain of Fpr3 has been shown to be important for binding with PP1 (a protein phosphatase) and helps in maintaining recombination checkpoint activity *in vivo* (Hochwagen et al., 2005). Fpr4, another yeast homolog of FKBP25, interacts with the

histone H3-H4 complex and facilitates nucleosome assembly in a similar manner as the other acidic histone chaperones characterized before. Fpr4 exhibits chaperone activity by its N-terminal acidic domain (Xiao et al., 2006). Furthermore, FK506 does not affect chaperone activity of Fpr4 which indicates that PPIase domain has no role in chaperone activity. Fpr4 targets the rDNA locus for its silencing. But rDNA silencing was shown to be independent of N-terminus of Fpr4, which suggests that the N-terminus of Fpr4 is not involved in gene silencing and it is only responsible for chaperone activity.

Fpr4 also regulates histone methylation. The first report towards this end came in 2006 by Nelson et al., where they showed that proline isomerization in histone protein can regulate its methylation, a new mechanism of histone modification. Fpr4 binds with the amino-terminal tail of histone by its nucleolin-like (NL) domain. This interaction places Fpr4's PPIase domain close to its two proline substrates P30 and P38 of H3, which catalyzes isomerization of proline H3P30 and H3P38 (Nelson et al., 2006). Furthermore, they demonstrated that P38 of H3 is critical for methylation of K36 of H3 by Set2, a well-known histone methyltransferase. Fpr4 mediated isomerization of P38, protects K36 from methylation by Set2 (Nelson et al., 2006).

This study showed a novel role of nuclear FKBP in histone modification. Such studies are limited to yeast and plants and the possible roles of FKBP25 as histone chaperone and histone modifier, if any, needs to be elucidated. Future study is required to understand the role of nuclear FKBP (like FKBP25, Fpr3, and Fpr4) in several nuclear events like ribosomal synthesis, chromatin remodeling, and cell-cycle regulation and also their role in the progression of cancer.

Figure 1.7: Possible roles of FKBP in nuclear events. FKBP may serve as a histone chaperone or a histone modifier. FKBP is also involved in transcription regulation by interacting with transcription factors. They might have a role in DNA methylation. FKBP can also participate in gene activation by changing the conformation of histone by prolyl *cis/trans* isomerization (adapted from Yli Yao review 2011).

The plant homologs of FKBP25 are shown to have a similar function as Fpr3 / Fpr4. *AtFKBP53*, an Arabidopsis FKBP, also possesses histone chaperone activity and causes repression of the rDNA expression (Li and Luan, 2010). The N-terminal domain of *AtFKBP53* is an acidic domain and was shown to be important for histone binding and chaperone activity (Li and Luan, 2010).

All the above studies advocate for the function of plant and yeast homologs of FKBP25 as histone chaperones and also suggests a possible function of FKBP25 as a histone chaperone. For such histone chaperone activity, FKBP25s need their N-terminal acidic domain but not the C-terminal conserved PPIase domain. Such acidic domains are not present in mammalian FKBP25s. However, human FKBP25 has a unique N-terminal domain but it consists of mainly basic residues which suggest it may have a role in histone chaperone.

1.3: A brief introduction of FKBP25

FKBP25, a member of FK506 binding protein, is a 25.2 kDa protein which binds with both rapamycin and FK506 but shows higher affinity for rapamycin. Like other FKBP25s, FKBP25 possesses a conserved FK506 binding domain (FKBD), also called the PPIase domain. In most of the FKBP25s, the FK506 binding domain is present at the N-terminal while FKBP25 has its FK506 binding domain at the C-terminal (Figure 1.2). The FK506 binding domain of FKBP25 shows high sequence similarity to FKBP12. PPIase activity of FKBP25, just like other FKBP25s, can be inhibited by FK506 and rapamycin. The unique feature of FKBP25 is its multifunctional hydrophilic N-terminal domain which embodies a helix-loop-helix (referred as HLH) motif. 38 % of the residues in the N-terminal domain of FKBP25 contain charged side chains. The N-terminal domain of human FKBP25 does not show significant sequence similarity with any human protein (Horowitz et al., 1994b). Full-length human FKBP25 contains 224 amino acids out of which 1-74 amino acids make up the N-terminal domain, amino acids 109-224 form FKBD and these two domains are linked by a 35 amino acids loop (Figure 1.8A). The amino acid sequence of full length FKBP25 is shown in Figure 1.8B. Although the function of FKBP25 in the cell is not fully characterized, FKBP25 has been shown to interact with several nuclear proteins (Figure 1.9).

Figure 1.8: Sequence of full-length FKBP25. The residues belong to HLH domain (1-74), flexible loop (74-109) and FKBD (109-224) are shown in red, green and purple color respectively.

Figure 1.9: Illustration showing interacting partners of FKBP25. FKBP25 can interact with different nuclear proteins either by its N-terminal domain or by C-terminal PPIase domain.

1.3.1 FKBP25: a nuclear localizing protein

Most of the FKBP family members identified to date reside in the cytoplasm to perform *cis* / *trans* isomerase activity. FKBP25 is the first mammalian FKBP which was found to be located in the nucleus (Jin and Burakoff, 1993). Jin and Burakoof also demonstrated the ability of FKBP25 to associate with casein kinase II and nucleolin in nuclear extracts. FKBP25 contains a nuclear localization sequence and several potential casein kinase II phosphorylation sites. As nuclear localization of a protein can be enhanced by casein kinase II phosphorylation, it was suggested that casein kinase II mediated phosphorylation of FKBP25 may have a role in nuclear localization of FKBP25. (Jin and Burakoff, 1993). Later it was shown that 62 % of FKBP25 localizes into the cytoplasm, 23% into the nucleus and 15 % into the nucleolus (Geoff et al., 2015). The binding of FKBP25 with nucleolin was shown to be dependent on rRNA which further demonstrated an involvement of FKBP25 ribosome biogenesis.

Furthermore, Leclercr et al. have shown that porcine high mobility group (HMG) II protein can interact with non-modified FKBP25 (Leclercq et al., 2000). HMG, a non-histone protein, mediates DNA binding of several proteins and thus helps in transcriptional regulation of several genes. It was suggested that FKBP25 together with HMG-II regulates the transcriptional activity of some genes. In an earlier report Rivière et al. demonstrated that FKBP25 can bind with DNA-affinity matrices, suggesting that FKBP25 can be a DNA binding protein (Riviere et al., 1993). All the above evidences advocate for a possible role of FKBP25 in DNA binding and transcriptional regulation of genes.

1.3.2 FKBP25: role in p53 pathway regulation

p53 is a transcription factor which gets induced by a variety of cellular stress conditions e.g. DNA damage. MDM2 is a key regulatory protein of p53 pathway as MDM2 mediates degradation p53. Once activated, in cellular stress condition, p53 gets rid of MDM2 to mediate growth arrest or apoptosis and also stimulate MDM2 auto-ubiquitylation and degradation (Toledo and Wahl, 2006). Ahn et al. have demonstrated that FKBP25 is a downstream gene to the p53 pathway and p53 can repress the expression of FKBP25 gene (Ahn et al., 1999). Later Ya-Li Yao et al. discovered FKBP25 as an interacting partner of MDM2 by yeast two-hybrid system. C-terminal FKBD of FKBP25 was shown to be important for MDM2 binding. As the N-terminal domain of FKBP25 contains a helix-loop-helix motif, one can speculate that some other unknown proteins can also interact with N-terminal of FKBP25 to mediated FKBP25-MDM2 interaction. None of the MDM2 deletion derivatives was able to interact with full-length FKBP25 and only full-length MDM2 showed interaction with FKBP25 in the pull-down assay, which suggests that optimal interaction with FKBP25 is mediated by multiple contacts (Ochocka et al., 2009). FKBP25 interaction with MDM2 stimulates auto-ubiquitination of MDM2 by an unknown mechanism and thus increases the level of p53 and its downstream gene p21 (Figure 1.11). Knockdown of FKBP25 results in decreased p53 followed by a p21 expression. These studies clearly show the connection between FKBP25 and p53 pathway. Further study using FKBP25 knockout cells or animals would broaden the understanding of the role of FKBP25 in p53 pathway and ultimately in the progression of cancer.

1.3.3 FKBP25: role in histone deacetylation

Involvement of acetylation and deacetylation of lysine residues at the N-terminus of histone protein has emerged as an important mechanism of regulation of gene expression. Histone deacetylase (HDAC) is an enzyme which removes an acetyl group from histone which leads to transcriptional repression. Several HDACs have been identified e.g. HDAC1, HDAC2, HDAC4, HDAC5, SIR2 like protein, maize HD2 protein etc. (Grunstein, 1997; Yao et al., 2011). HD2 does not show homology to other class of HDACs but surprisingly shows similarity to FKBP-type PPIase. Association of FKBP25 with other proteins like CK-II, nucleolin and HMG-II indicated a possibility of FKBP25's role in

transcriptional regulation. Yang et al. have demonstrated that FKBP25 performs a histone deacetylase function which leads to gene repression (Figure 1.11). They advocated that it was not the intrinsic property of FKBP25 to deacetylate histone; rather interaction of FKBP25 with HDAC1/2 mediates histone deacetylase function (Yang et al., 2001). The precise mechanism and regulation of FKBP25-HDAC interaction need to be elucidated further. It is also important to identify the other components of the FKBP25-HDAC complex.

Apart from HDAC, a recent study revealed that FKBP25 can interact with core histone of the nucleosome, which suggested that FKBP25 could be a crucial component in the process of DNA repair, chromatin remodeling or gene regulation (Gudavicius et al., 2014).

Figure 1.10: Illustration shows the different roles of FKBP25 in the nucleus. FKBP25 interacts with HDAC1/2 through its N-terminal HLH domain which increases histone deacetylation and transcriptional repression of the gene. Its N-terminal domain also binds with YY1 to increase transcriptional repression by YY1. The FKBD of FKBP25 interacts with MDM2 and increases auto-ubiquitination of MDM2. Auto-ubiquitination of MDM2 results in its degradation and hence increases the p53 levels.

1.3.4 FKBP25: role in regulation of transcription factor

Yin Yang 1 (YY1) is a ubiquitous and multifunctional transcription factor and it belongs to polycomb group protein family. It acts as both a repressor and activator to regulate a list of genes involved in cancer development and progression e.g. c-myc, c-fos, ERBB2, E1A and p53. It also interacts with a number of proteins like Rb, Mdm2, Ezh2, caspases, FKBP12 and HDACs [reviewed by (Deng et al., 2010)]. YY1 has been shown to be involved in a myriad of biological processes like cell proliferation, differentiation, replication, and embryogenesis.

YY1 is made up of 414 amino acids, and it bears mainly an N-terminal transcription activation domain and two C-terminal transcription repression domains (Figure 1.11). The C-terminal transcription repression domain is composed of four zinc fingers and thus forms DNA binding domain.

Figure 1.11: Domain organization of YY1 protein. C-terminal DNA binding domain consists of four zinc fingers.

The co-crystal structure of C-terminal DNA binding domain (zinc finger domain) of YY1 in complex with DNA was solved by (Houbaviy et al., 1996). The structure revealed that four zinc fingers of YY1 wrap the DNA through the major groove (Figure 1.12). YY1 recognizes and binds with DNA through its second and third zinc fingers. First zinc finger was found to be loosely bound to DNA.

Depending upon the context, YY1 can perform either transcription activation or repression by a different mechanism. The binding affinity of YY1 is relatively low (in the micromolar range) with respect to other transcription factors (in nanomolar range), which

suggested that YY1 may require other co-regulator proteins to perform gene activation or repression. Although the mechanism of gene activation or repression by YY1 is not fully elucidated, several models have been proposed to explain this process. It was suggested that in some cases, the co-activator protein could compete for the DNA binding site of YY1 for gene repression which eventually leads to gene activation. Another model suggests that direct interaction of co-regulator proteins with YY1 could bring some structural changes into YY1 which could be important for the function of YY1. YY1 could also recruit corepressors which could facilitate chromatin remodeling to help in gene repression.

Figure 1.12: Co-crystal structure of YY1 bound to DNA (PDB ID- 1UBD). The structure shows that four zinc fingers of DNA binding domain of YY1 wrap around DNA through the major groove of DNA.

It was demonstrated that YY1 could also directly interact with FKBP25 (Yang et al., 2001). YY1 also interacts with the PPIase domain of FKBP12 but in the case of YY1-FKBP25 interaction, YY1 interacts only with unique N-terminal HLH but not with the PPIase domain of FKBP25. As PPIase domain of FKBP25 is not involved in the FKBP25-

YY1 interaction, rapamycin and FK506 do not affect this interaction. It was also shown that residues of YY1 from 300 to 333, a region that comprises one and a half zinc fingers (critical for YY1's sequence-specific DNA binding), is essential for YY1-FKBP25 interaction (Yang et al., 2001). Further study showed that FKBP25–YY1 interaction, by an unknown mechanism, increases the DNA binding ability of YY1 and thus increases the repression activity of YY1 (Yang et al., 2001) (Figure 1.11). A separate study has demonstrated that acetylation or deacetylation of YY1 can regulate its DNA-binding activity [reviewed in (Deng et al., 2010)]. So it is possible that FKBP25 interacts with HDACs to promote deacetylation of YY1 and thus increases YY1's DNA binding capacity. Another possibility is that FKBP25 interacts with YY1 to increase its DNA binding activity by bringing some structural changes in YY1. Another possibility is that YY1, DNA and FKBP25 form a ternary complex and thus stabilizes YY1 onto DNA. These findings have raised a number of open questions. How does FKBP25 influence the DNA-binding activity of YY1? What is the biological significance of such increased DNA-binding activity of YY1? Does FKBP25 change the transcriptional activity of YY1 target genes? Is there any transcription factor, other than YY1, which can be regulated by FKBP25?

1.3.5 Structural features of FKBP25

The solution structure of N-terminal domain was solved by Helander et al., which showed that this domain bears five alpha helices which are joined by short loops (PDB ID -2KFV) (Figure 1.13). Although the sequence of an N-terminal domain of FKBP25 does not match with any human protein, the structure of the N-terminal domain of FKBP25 has some similarity to a subdomain of the HectD1, a known E3 ubiquitin ligase.

The crystal structure of FKBD of FKBP25 bound to rapamycin was solved by Liang et al. Although the crystal structure of free FKBD25 is not solved yet, FKBD25-ramamycin complex structure showed that this is similar to FKBD of other FKBP. FKBD25 also bears five antiparallel beta sheets and a central alpha helix. The rapamycin binds in the core of the hydrophobic pocket located in FKBD25 (Figure 1.14).

Figure 1.13: The solution structure of N-terminal HLH domain of FKBP25 (PDB ID-2KFV). The structure is shown in cartoon representation by rainbow coloring scheme and it shows five α helices joined by short loops. All α helices labeled and the N-terminal and c- terminal are labeled as N and C respectively.

Figure 1.14: The crystal structure of C-terminal FKBD of FKBP25 in complex with rapamycin (PDB ID-1PBK). The structure is shown in rainbow coloring with all the β sheets, α helix and loop labeled.

Although the structure of N-terminal HLH domain and FKBD in complex with rapamycin are solved, neither the structure of FKBD in complex with FK506 drug nor the structure of full-length FKBP25 has been solved yet. In several studies, it was shown that full-length FKBP25 is required for its full functionality. For example, it was shown that neither N-terminal HLH domain, not the C-terminal FKBD was able to interact with nucleolin but full-length FKBP25 could interact with nucleolin. To understand the topology of these two domains in full-length FKBP25 and also to understand how these two domains may cooperate with each other to facilitate interaction with other proteins, it becomes imperative to solve the structure of full-length FKBP25. This would also shed light on how the long flexible loop connects these two domains. Once the structure is elucidated, it would be easier to map the binding sites of other proteins, DNA and drugs on FKBP25. In Pin1, another PPIase protein, it was shown that two of its domains could interact with each other and such interaction was found to be very important for the PPIase activity of this protein (Bourn et al., 1994). As FKBP25 has two unrelated domains which are linked through a long loop, it would be interesting to explore any possible domain-domain interaction in FKBP25. Towards this end, we have solved the solution structure of FKBP25 and showed the presence of domain-domain interaction between HLH and FKBD of FKBP25.

1.3.6 Interaction of FKBP25 with FK506 and rapamycin.

One of the interesting features of FKBP25 is its differential binding affinity to rapamycin and FK506. In all other FKBP25s, the binding affinity to FK506 and rapamycin is almost the same. FKBP25 shows 200 fold higher binding affinity to rapamycin and that is why FKBP25 is considered mainly as a rapamycin binding protein. Some of the possible explanations for such difference in binding affinity were proposed by Jun Liang et al but due to lack of the availability of FKBD25-FK506 complex structure, this question remained elusive. Several derivatives of FK506 and rapamycin have been developed and understanding the mechanism of such differential binding would help to develop novel drugs. Towards this end, we have solved the crystal structure of FKBD25-FK506 complex to shed light on this differential binding behavior.

1.4: A brief introduction of DNA binding proteins

Binding of proteins with DNA can mainly be divided into two categories: sequence-specific binding and sequence non-specific binding.

1.4.1 Sequence-specific DNA binding

Sequence-specific DNA binding proteins interact with DNA mainly through electrostatic, H-bond, and hydrophobic interactions. The side chain of amino acids like Glutamine, Asparagine, Arginine, and Lysine, can form a hydrogen bond with NH₂ and X=O of base pairs of DNA. These interactions could be mediated through the major or minor groove of DNA. Several structural motifs of protein help for sequence-specific binding of the protein with DNA. Some of these motifs are as follows

(a) Helix turn helix. In a helix turn helix motif, there are 2 short helices of 7-9 amino acids which are separated by a 3-4 amino long non-helical structure (Figure 1.15). Examples are Cro and cI proteins of Lambda, RPA1 and Lac-Z of *E.coli*.

Figure 1.15: Interaction of DNA with Cro protein which bears a helix-turn-helix domain.

(b) Zinc finger: It is a finger-shaped motif which requires zinc ion to stabilize the fold. Usually, 2 histidines from helix and 2 cysteines from a loop coordinate with a zinc to form zinc finger domain (Figure 1.16). The helix of the zinc finger interacts with DNA and the zinc finger is only important to maintain the protein fold, not for DNA binding. Some of the examples are histone acetyltransferase, Zif268, Myt1, and YY1 etc.

Figure 1.16: Zinc finger domain from Zif268 protein (PDB-1AAY). Protein is shown in green color while the Zinc is shown in purple color.

(c) Leucine Zipper proteins: Two alpha helix bearing repeats of leucines after every 7 amino acids form a dimer called a Leucine Zipper (Figure 1.17). It recognizes a specific sequence of DNA and bind to either side of DNA at the major groove. Examples are c-fos, c-jun, myc, max 9 etc

Figure 1.17: Interaction of DNA with c-fos protein which shows Leucine Zipper motif (PDB ID- 1FOS)

1.4.2 Sequence non-specific DNA binding

Sequence non-specific or sequence-independent DNA binding protein interacts with any sequence of DNA without recognizing the sequence of DNA. The protein-DNA interaction is mainly mediated by the electrostatic interaction between the phosphate backbone of DNA and the positively charged residues (like Arginine, Lysine, and Histidine). There are several examples of such interaction and few of them are LrpC, HMG1 and Rab1 (Bustin, 1999; Tapias et al., 2000; Thomas, 2001). One the most common examples of such interactions are binding of histone protein with DNA to form a nucleosome (Sandman et al., 1998). Sequence-independent DNA binding can facilitate DNA bending, DNA repair, chromosome remodeling etc. (Bustin, 1999; Tapias et al., 2000; Thomas, 2001).

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 Chemicals

PMSF	Sigma (Saint Louis, MO, USA)
TCEP	Soltec Ventures (Beverly, MA, USA)
DTT	Gold BioTechnology (Saint Louis, MO, USA)
BME (2-mercaptoethanol)	Sigma (Saint Louis, MO, USA)
Ni ²⁺ - NTA High performance	GE Healthcare (Uppsala, Sweden)
LB media	BD (Sparks, MD, USA)
Electrophoresis chemicals and reagents (Agarose, SDS, Glycine, APS etc.)	Bio-Rad (Hercules, CA, USA)
Antibiotics	Sigma and Gibco (Invitrogen)
IPTG	Gold Biotechnology (USA)
BSA	Sigma and Bio-Rad

2.1.2 Molecular biology materials

Plasmid DNA (pE-SUMO, T7, Kan)	LifeSensors (Marvern, PA, USA)
Primers	Sigma (USA)
Pfu and T4 DNA Polymerase	Fermentas (Glen Burnie, MD, USA)
BsaI, BamHI and XhoI	New England Biolabs (NEB)
T4 DNA ligase	Fermentas and NEB
Miniprep Plasmid kit	Axygen (Union City, CA, USA)
Escherichia coli expression strains	BL21 (DE3) (Novagen)

2.1.3 Chromatography

2.1.3.1 Affinity chromatography

Lactose agarose	Sigma
Ni ²⁺ - NTA High performance	GE Healthcare (Uppsala, Sweden)
Gel filtration	
Superdex 200 HR (10/30)	GE Healthcare (Uppsala, Sweden)
PD-10 Desalting	GE Healthcare (Uppsala, Sweden)

2.1.3.2 Instruments and accessories

Akta FPLC UPC-900	GE Healthcare (Uppsala, Sweden)
Syringes, needles and accessories	BD Biosciences

2.1.3.3 Protein concentration devices and estimation

Amicon ultra (3 kDa MCO)	Millipore (Co-cork, Ireland)
Bio-Rad Protein assay	Bio-Rad (Hercules, CA, USA)

2.1.4 Other instrumentation

PCR Thermocycler	Applied Biosystems (USA)
NanoDrop Spectrophotometer ND 1000	NanoDrop Technologies (USA)
FACS Calibur	BD Biosciences

2.1.5 Computer software

Vector NTI 10.3.0	Invitrogen
Quantity One	Bio-Rad

2.1.2 Media

Different types of media were used for expression and purification of proteins in *E.coli*. Luria-Bertani (LB) media was mainly used for the expression of the unlabelled recombinant protein in *E.coli*. 5 gm NaCl, 5 gm yeast extract and 10 g of bacto tryptone were dissolved in 1 litre of water and autoclaved at 121° C for 15 mins was used for the study. To make LB agar plate 2% agar was added to the medium before autoclaving. Different antibiotics were added to the medium as required before bacterial culture.

2.1.3 Antibiotic stock

Kanamycin was dissolved in water at concentration 30 mg/ml and sterile filtered. Aliquots were stored in the refrigerator and used when needed. Ampicillin 100 mg/ml, carbenicillin 100 mg/ml, chloramphenicol 20 mg/ml in ethanol were prepared accordingly and used for bacterial culture.

2.1.4 Buffers and solutions

Buffers for running DNA gel electrophoresis

2.1.4.1 50 × TAE (Tris-acetate-EDTA) (DNA agarose gel running buffer)

242 g Tris base, 57.1 ml glacial acetic acid, 100 ml 0.5 M EDTA were dissolved in 800 ml dd H₂O and the pH was adjusted to 8.0. The solution was topped up to 1 L with dd H₂O.

2.1.4.2 TE (Tris-EDTA) buffer

10 mM Tris-base and 1 mM EDTA were dissolved in dd H₂O and the pH was adjusted to 8.0 by HCl.

2.1.4.3 Agarose solution for running gel electrophoresis

The required amount of agarose was dissolved in 1X TAE buffer and the solution was microwaved to dissolve. The solution was then stored at 65°C for future use. The solution prior to use is mixed with ethidium bromide and poured into casting chamber for solidification.

2.1.4.4 Ethidium bromide solution

Ethidium bromide tablets (GE healthcare) were dissolved in distilled water to produce a stock concentration of 10 mg/ml.

2.1.4.5 6X DNA loading dye

0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 40% (w/v) sucrose were dissolved in dd H₂O. The loading dye was stored at 4 °C before use.

Buffers used for protein expression and purification

2.1.4.6 Preparation 1M IPTG solution

7.1 g IPTG was dissolved in 30 ml of dd H₂O and sterile filtered. The solution is stored in small aliquots at -20°C and is used for induction of bacterial cultures for protein production.

2.1.4.7 Resuspension buffer (for bacterial cell pellet)

20 mM phosphate buffer, 0.5 M NaCl, 1 mM PMSF were dissolved in double distilled H₂O. The pH of the solution was adjusted to 7.8

2.1.4.8 Washing buffer

20 mM phosphate buffer pH , 1 M NaCl, and 20 mM imidazole, 1 mM PMSF were dissolved in double distilled H₂O. The pH of the solution was adjusted to 7.4. Several other washing buffers with increasing concentration of imidazole were often used depending on purification profile of protein of interest.

2.1.4.9 Elution buffer

20 mM phosphate buffer, 0.5 M NaCl, 0.4 M imidazole and 1 mM PMSF were dissolved into double distilled H₂O. The pH of the solution was adjusted to 7.0.

Buffers for running SDS-PAGE gel

2.1.4.10 5X buffer for running SDS-PAGE electrophoresis

15.1 gm of Tris-base, 72 g glycine and 5 g of SDS were dissolved carefully to 1 litre distilled water. The solution can be stored at room temperature.

2.1.4.11 2X SDS-PAGE loading dye

100 mM Tris-Cl, 4% SDS, 0.2% Bromophenol blue, and 20% (v/v) glycerol were dissolved in dd H₂O and the pH was adjusted to 6.8 using HCl. Prior to use, β -mercaptoethanol was added to a concentration of 10 mM. The solution was stored at -20 °C before use.

Buffers and reagents for western blot analysis

2.1.4.13 Transfer buffer

2.9 g glycine and 5.6 g Tris were dissolved in 1litre distilled water containing 20 % methanol. 1 ml of 10 % SDS was added to it and used as transfer buffer.

2.1.4.14 Tris-buffered saline (1×TBS)

20 mM Tris and 500 mM NaCl were dissolved in dd H₂O and the pH was adjusted to 7.5 using HCl or alternatively 20 X TBS (commercially available) was diluted to 1X TBS with distilled water.

2.1.4.15 Washing buffer (TTBS)

1X TBS containing 0.05% tween 20 as detergent.

2.1.4.16 Blocking buffer

5% Non-fat dry milk in TTBS.

2.1.4.17 Antibody buffer

5% BSA in TBTS containing antibody at appropriate dilution (Primary) and 5% Non-Fat dry milk in TTBS containing antibody (secondary) at appropriate dilution.

Table 2.1 List of primers and their sequence

Construct	Restriction site	Oligonucleotide sequence
Full length	Forward (BsaI)	5'ATAGGTCTCAAGGTATGGCTGCCGCTGT3'
FKBP25	Reverse (BamHI)	5'CGCGGATCCTTAGTCAATGTCAACCAGT 3'
FKBP25	Forward (BsaI)	5'ATAGGTCTCAAGGTATGGCTGCCGCTGT 3'
(1-90 aa)	Reverse (BamHI)	5'GCGCGGATCCTTACAGCTTGACGTTTTTAAC 3'
FKBP25	Forward (BsaI)	5'ATAGGTCTCAAGGTATGGCTGCCGCTGT 3'
(1-109 aa)	Reverse (BamHI)	5'TCGCGG ATCCTTATTCGTCCAGGGTTTCTTC 3'
FKBP25	Forward (NdeI)	5'GCCCATATGCCGAAATATACGAAGTCTGT3'
(109-224 aa)	Reverse (XhoI)	5'ACACTCGAGGTCAATGTCAACCAGTTCCA 3'

2.2 Methods

2.2.1 Agarose gel electrophoresis for DNA

Required percentage (usually 1%) of agarose gel was cast in casting tray and allowed to solidify. 4 µl of ethidium bromide solution (10 mg/ml) or gel red dye was added to the solution and mixed uniformly in order to visualize the DNA bands. After solidification 100 to 500 ng DNA samples were added to the wells and the samples were electrophoresed at 80 volts for 25-30 mins. The gel was then seen under UV light to visualize the bands. Different DNA marker (1kb or 100bp DNA marker) bands were also run simultaneously to estimate the size of the bands.

2.2.2 Determination of DNA concentration

The concentration of DNA can be determined using the spectrophotometric method as well by comparing with DNA marker standards. DNA absorbs light of certain wavelength so that light of 260 nm passing 1 cm through DNA at 50 ug/ml concentration (in water) has an absorbance of 1.0. Multiplying OD (260 nm absorbance) with 50 ug/ml will give DNA concentration (inside the cuvette) and a further multiplication with the dilution factor will give DNA concentration in the sample.

A good quality DNA sample should have an A260/A280 ratio of 1.7-2.0 and an A260/A230 ratio of greater than 1.5, but since the sensitivity of different techniques to these contaminants varies, these values should only be taken as a guide to the purity of the sample. For an accurate measurement, the A260 value must lie between 0.1 and 1, so dilution of concentrated samples may be required.

The concentration of DNA samples can also be roughly estimated by comparing the intensity of DNA standard used. So the concentration of DNA was determined using nano drop.

2.2.3 Competent cell preparation

Generally, chemically competent host cells (*E.coli* strain DH5 α , Bl21 (DE3) etc) were used. A single colony was picked up from the bacterial plate and allowed to grow till the OD of the culture reaches 0.5-0.6. The cells were then spun down and resuspended in ice cold sterile 100 mM MgCl₂. Henceforth, all the operations were conducted at 4°C. The cells were again spun down and resuspended in ice cold sterile 100 mM CaCl₂. The cells can be suspended for 2 hours to become competent. Alternatively, cells can also be suspended overnight. The suspension is then aliquoted in small volumes after addition of 50% glycerol as a cryoprotectant and stored at -80°C for future use.

2.2.4 Cloning of the gene into bacterial/mammalian expression vector

Human FKBP25 gene, cloned in pUC57 vector, was obtained from GeneScript. Human YY1 gene was obtained from PlasmID Repository at Harvard Medical School. In order to clone genes in SUMO expression vector, forward primers with *BsaI* restriction site were designed. The restriction site for reverse primers was chosen from the MCPs of SUMO vector. For cloning in other expression vectors, primers were designed with one of the restriction site chosen from the MCPs of the respective vectors for each of the forward and reverse primers. Different properties of primer were checked by oligo-analyser, an online software from Integrated DNA Technologies. Genes were amplified by polymerase chain reaction (PCR). The maps of the clones of FKBP25 in SUMO or pET29b are shown in Figure 2.1.

Figure 2.1 (A) vector map of pSUMO expression vector having FKBP25 gene cloned in. (B) Vector map of pET29b with FKBD25.

Amplified gene and the vector were digested with two enzymes in a sequential manner. Then the digested products were cleaned up to get rid of restriction enzyme and then were subjected to the ligation reaction. After ligation, ligated product was transformed into competent DH5 α cells. Positive clones were selected by colony PCR and confirmed by DNA sequencing. Below is the detail of different steps used for cloning.

2.2.4.1 Polymerase chain reaction (PCR) amplification

PCR is one of the basic reactions in biology, extensively used for amplification of the gene of interest for different cloning reactions. In our case, for recombinant gene cloning, we have used two different DNA polymerases for our purpose- the *Pfu* DNA polymerase, which have proofreading activity, was used for amplification of gene of interest and the *Taq* DNA polymerase which lacks the proofreading activity, was used for colony PCR reactions to check the success of cloning reactions. A total of 50 μ l of PCR reaction mix was prepared on ice with the constituents listed below

Table 2.2 Components of PCR reaction mixture using *Pfu* polymerase

Reagent	Volume in μl	Final concentration
10x buffer	5	
Forward primer (10 μM)	2.5	0.5 μM
Reverse primer (10 μM)	2.5	0.5 μM
dNTPs (10 mM)	0.5	0.1 mM
<i>Pfu</i> polymerase	0.5	
Template DNA	1.25	
MilliQ water	33.75	
Total	50	

Table 2.3 Components of PCR reaction mixture for *Taq* polymerase

Reagent	Volume in μl	Final concentration
10x buffer	5	
MgCl ₂ (25 mM)	4	2 mM
Forward primer (10 μM)	2.5	0.5 μM
Reverse primer (10 μM)	2.5	0.5 μM
dNTPs (10 mM)	0.5	0.1 mM
<i>Taq</i> polymerase	0.5	
Template DNA	1.25	
MilliQ water	33.75	
Total	50	

The components were mixed and put in a thin walled 0.2 ml PCR tube and incubated on GeneAmp 9700 PCR system (Applied Biosystems). Following steps are used for the reaction.

Table 2.4 Condition for PCR reaction

Cycle	Temperature	Time
Initial denaturation	95°C	5 min
Denaturation	95°C	30 sec
Annealing	65-70°C	30 sec
Extension	72°C	1-2 min
Final extension	72°C	7 min
End of the cycle	Cool down to 4°C	

For the PCR condition, the annealing temperature and time depends on the T_m of the primer used; and the extension time depends on the type of polymerase used as well as the length of the gene used. Generally, 25-30 thermal cycles were used for the amplification. After the reaction, the amplification of the product bands was checked by running agarose gel electrophoresis.

2.2.4.2 Gel extraction of DNA

The DNA sample was run on agarose gel for separation of the bands. The gel was then visualized under a low-intensity UV lamp, the desired gel band cut out quickly using a blade. Over exposure of UV light might cause mutation in DNA amplicon. The DNA was then extracted out of the gel using gel extraction kit (commercially available) according to the manufacturers' instructions.

2.2.4.3 Restriction digestion

Purified PCR product or plasmid DNA was digested with restriction enzymes for cloning reactions. The DNA was mixed with the required enzyme and incubated at the required temperature in the buffer stated by the manufacturer. Generally, the reaction was

performed at 37°C for 3 hours in order to ensure the completion of the digestion. In some case when the buffers for two restriction enzymes were not compatible, sequential digestion was performed and in another case when buffers were compatible we performed double digestion using both enzymes together for digestion. After the completion, the product was purified using commercially available kits.

2.2.4.4 Ligation

Digested PCR product and digested plasmid vectors were ligated for insertion of the gene of interest. T4 DNA ligase was used for the purpose. The vector and insert were mixed in different ratios *e.g.* 1:1, 1:2, 1:3 for the reaction. Ligation was performed at 16°C overnight. Different conditions were used depending upon the situation. The ligation product was then transformed into competent cells (DH5 α) and incubated for the appearance of colonies.

2.2.4.5 Colony PCR reaction

Colony PCR reaction was used to distinguish between positive and false positive colonies. Around 10 colonies were picked and used for colony PCR using the same forward and reverse primers which were used for PCR amplification of the gene. Amplified product was analysed on 1% agarose gel. Appropriate positive and negative controls were made. After picking positive clone, 5 ml of culture was grown overnight and then plasmid was isolated and sent for sequencing.

2.2.4.6 DNA sequencing

All DNA sequencing reactions were performed using required set of primers, from 1st Base, Singapore. The results obtained from the sequencing were matched with the original gene sequence using Vector NTI software (Invitrogen).

2.2.5 Site-directed mutagenesis

Site-directed mutagenesis was performed as reported previously. For single amino acid substitution, the primers were designed in such a way that it contains the mutation (changed base) in the middle of the primer. The condition for the PCR is mentioned as

below. A plasmid containing wild-type gene of interest was used as a template for PCR amplicon. After PCR amplification, the amplicon was purified using PCR clean up kit. Later to digest template plasmid, PCR amplicon were digested with *DpnI* for 3 hours at 37°C. Digested product was transformed into XL-blue competent cells. Few colonies were picked and used for plasmid preparation and the mutants were confirmed by sequencing them.

2.2.6 Concentration of protein samples

Large volume pure protein samples were concentrated using an Amicon ultra concentrating device. The membrane of the device is first wetted with the sample buffer to avoid sample sticking. Samples were next poured into the top chamber and centrifuged in a swing bucket rotor at 3700 rpm in a cold centrifuge. Buffer gets drained out across the membrane and protein gets concentrated at the top portion of the device.

2.2.7 Protein concentration determination

BioRad reagent for protein concentration is used for the assay. The reagent is diluted 1:4 with deionized water. Spectrophotometer wavelength is set at 595 nm. A standard curve with a known protein (BSA) was first prepared. In order to obtain the standard plot, increasing amount of the protein (BSA) was mixed with 1 ml of Bradford solution and absorbance recorded. A plot of concentration versus absorbance was prepared. The concentration of the unknown protein sample was determined by recording its absorbance and then by extrapolation on the standard curve. NanoDrop spectrophotometer was used for protein concentration determination.

2.2.8 SDS-gel electrophoresis

Denaturing SDS-PAGE is used to check the purity of protein samples. The samples were collected during each step of a process of purification were mixed with 2X-SDS gel loading dye and heated at 95°C for 2 mins to denature the samples. These were next loaded to SDS-PAGE gel and run at 100 V for around 90 mins or till the dye front reaches the terminal mark. The gel was then stained using the staining solution. After about 40 mins the stained gel was transferred to destaining solution to visualize the protein bands. Once

the gel was nicely visualized a scanned image of the gel collected using the densitometric scanner and stored for future reference. Gels were also preserved after drying in between cellophane sheets.

2.2.9 Expression of recombinant proteins in *E. coli*:

2.2.9.1 Plasmid extraction

Plasmids containing the cloned gene were extracted using AxyPrep Plasmid MiniPrep kit (Axygen) following the protocol provided by the manufacturer.

2.2.9.2 Transformation of competent *E. coli* cells

Competent BL21 (DE3) cells (Novagen) frozen at -80°C were used for expression of proteins. Cells in 1.5 ml tubes were thawed on ice and 1-2 µl of previously extracted plasmid DNA was added and gently stirred with the tip of a pipette or finger-flicked to prevent damage to fragile competent cells. This mixture was incubated on ice for 30 minutes. The tubes were then placed into a water bath at 42°C for 55 seconds then placed back on ice for 5 minutes to reduce cell damage. 1 ml of LB broth (without antibiotic) was added. Tubes were incubated for 1 hour at 37°C before centrifugation at 13,200 rpm for 10 minutes. 900 µl of supernatant was discarded. The pellet was resuspended in the remaining 100 µl of supernatant and spread on LB plates (with kanamycin [US Biological] added). Plates were incubated at 37°C overnight and colonies picked 12 - 16 hours later.

2.2.9.3 Test for induction of recombinant proteins

2-3 random colonies of transformed BL21 (DE3) were picked and inoculated into 10 ml of LB broth containing kanamycin. This starter culture was grown overnight at 37°C and 1ml of this starter culture were subcultured into 100 ml of LB broth containing kanamycin. The culture was incubated at 37°C until the optical density reached 0.6; the exponential phase of the bacterial growth curve. 1ml of culture was aliquoted and used as the un-induced sample. To induce protein expression, 0.2 mM of IPTG (GoldBio Technology) was added before incubation at 25°C for 3 hours. 1ml of induced and un-induced cells were harvested by centrifugation and resuspended in 50µl of 1×PBS. Lysed cells were heated for 5 min at 95°C on a heat block. 10 µl of both induced and uninduced

cell lysate were loaded into 12% SDS gel with suitable protein marker. Gel was stained with Coomassie blue and analysed.

2.2.9.4 Test for solubility of recombinant proteins

The solubility of recombinant protein was investigated in two different resuspension buffers. The composition of the buffer is listed below.

Table 2.5 List of buffer used for lysis of cells

Resuspension Buffer	Component
Phosphate buffer	20 mM phosphate (pH 7.8), 0.5 M NaCl
Tris buffer	20 mM Tris (pH 7.5), 0.5 M NaCl

100 ml of IPTG-induced cells were harvested by centrifugation at 8,000 rpm for 15 minutes and the supernatant was discarded. 40 ml of H₂O was added to the resuspended cell pellets which were then transferred to a 50ml falcon tube before centrifuging at 4,500 rpm for 30 minutes. Supernatant was discarded and 20 ml of each resuspension buffer (Phosphate buffer and Tris buffer) was added to each pellet. Each sample was sonicated for 10 minutes (pulse: 3.0 seconds on, 2.0 seconds off). During sonication and subsequent steps, the sample was kept on ice at all times to prevent degradation of the protein of interest. The lysate was spun down at 18,000 rpm for 30 minutes at 4°C and the supernatant was transferred into a clean 50ml falcon tube. The pellet was resuspended into 1M DDT. Both pellet and supernatant fractions were mixed with loading dye and loaded into 12% SDS gel. The appearance of the band corresponding to recombinant protein in supernatant fraction was an indication of solubility of the protein.

2.2.10 Purification of recombinant protein:

2.2.10.1 Affinity chromatography via poly-histidine tag system using Ni-NTA column

After confirming the induction and solubility of the recombinant protein, purification was done by Ni-NTA column affinity chromatography. Cells were induced and lysed as mentioned above and the supernatant was transferred into a Ni-NTA column

(Bio-Rad) containing equilibrated Ni-NTA beads (GE Healthcare). The column was washed with 20 column volume of a washing buffer [20 mM phosphate (pH 7), 1M NaCl and 20 mM imidazole]. Finally, the recombinant protein was eluted with elution buffer [20 mM phosphate (pH 6.5), 0.5M NaCl and 400 mM imidazol].

2.2.10.2 Desalting of protein sample/Buffer exchange of samples

Buffer dialysis was carried out to replace the elution buffer from Ni-NTA purified protein with a new buffer. In the case of recombinant proteins with SUMO tag, it's important to remove imidazole from the buffer, because imidazole interferes with the protease activity of SUMO protease which is used to cleave the SUMO fusion tag. The buffer was dialysed using a concentrator (Millipore) or by gel filtration (PD10 from GE Healthcare). 10 ml of diluted protein was concentrated to 1 ml by spinning at 3000 rpm. 1ml of concentrated protein was diluted with new buffer to make a final volume of protein around 10 ml and diluted protein was concentrated again. It was repeated 3 times and the protein was concentrated to desired volume and concentration. For buffer exchange by PD-10, the column was washed with 25 ml of 20% ethanol followed by 25 ml of new buffer for washing and equilibration of column respectively. Later 2.5 ml of concentrated protein was added to PD-10 and was allowed to drip old buffer and protein was eluted after adding 3.5 ml of new buffer. The new buffer conditions were 20 mM phosphate buffer (pH 7.0) and 100 mM NaCl.

2.2.10.3 SUMO digestion and purification of protein without Sumo tag

For the protein purified from 1 litre of bacterial culture, 40 µl of SUMO protease (Invitrogen) was used and the protein was allowed to digest overnight at 4°C or at room temperature for 1 hour. Digested proteins without the SUMO and polyhistidine tag were purified using a Ni-NTA column and collected in the flow-through and wash fractions. The elution fraction, which contained the SUMO tag and other non-specific bands, was discarded. Purified protein was run on a 12% SDS-PAGE to analyse purity of protein. Gels were stained with Coomassie Brilliant Blue.

2.2.10.4 Fast protein liquid chromatography (FPLC)

Once tagged protein samples were enriched using affinity chromatographic method, it can be further purified using gel filtration chromatography or other chromatographic methods. Different gel filtration columns from Amersham biosciences were used for purification of some of the proteins mentioned in the thesis and the process was performed in an ACTA FPLC workstation. The choice of buffers for the protein sample depends on the isoelectric point of the sample. A number of additives were also often used with the aim of increasing stability of the proteins. The sample fractions containing relatively purified protein were concentrated to a volume of 2 ml using a concentrator. It was next injected to the sample loop of the instrument. The progress of the chromatographic process is controlled and monitored using a computer device and software provided by the manufacturer (Unicorn, Amersham biosciences). Fractions containing protein were detected using an online absorbance detector (measures absorbance at 280 nm) and a conductivity detector; collected using a fraction collector. Samples were analyzed for purity using SDS-gel electrophoresis and used for further downstream applications.

2.2.11 Molecular weight determination using gel filtration

The gel filtration analysis is also another way of checking the molecular weight of proteins. The unknown molecular weight can be calculated from a standard curve drawn with known molecular weight proteins. The native molecular weight standard from BioRad was used in the calculation of molecular weight. The standard sample contain the following proteins: thyroglobulin (670 kDa); R-globulin (bovine) 158 kDa, Ovalbumin (Chicken) 44 kDa, Myoglobin (Horse) 17 kDa and Vitamin B12 1.3 kDa; after running the standards in the sizing column, the V_o (Void volume) and V_t (Total volume) of the column can be obtained. Based upon the V_e (Elution volume) of the standards the relationship between $k_{av} = (V_o - V_e) / (V_t - V_e)$ should be linear. Using this relationship, the molecular weight of the unknown protein sample can be calculated according to its V_e .

2.2.12 Regeneration of Ni^{2+} -NTA agarose

Ni^{2+} -NTA agarose can be regenerated after a single use and can be used almost as new ones. The following steps are usually followed for regeneration of Ni^{2+} -NTA agarose.

- i) The column is washed with 2 volumes of 6 M guanidine hydrochloride in 0.2 M acetic acid solution.
- ii) Washed with 2% SDS one volume
- iii) One volume of 25% ethanol
- iv) One volume of 50% ethanol
- v) One volume of 75% ethanol
- vi) One volume of 100% ethanol
- vii) One volume of water.
- viii) Five volume of 100 mM EDTA pH-8.0.
- ix) Regenerate with 2 volume of 100 mM NiSO₄.
- x) Wash with 1 volume of water
- xi) Resuspend in 20 % ethanol to make 50% slurry.
- xii) The slurry can be stored in cold and used when necessary.

2.2.13 Western blotting experiment

The western blotting is an analytical technique used to detect specific proteins in a given sample of tissue homogenate or extract. 10 to 30 µg of protein samples were first resolved using 12 % SDS-gel electrophoresis. A prestained molecular weight marker (dual color marker) was also used simultaneously to monitor the transfer of proteins. The protein samples were next transferred to nitrocellulose or a PVDF membrane. Presently, two methods are widely used for the transfer reaction, the wet-transfer method and the semi-dry method. In the former, the transfer reaction takes place in the presence of buffer solution whereas in the latter method the transfer takes place in between two wet pieces of filter paper (wetted in the buffer). In the preparation of the filter paper-gel-membrane-filter paper cassette, it is very important to remove any air bubble in between in order to ensure an efficient transfer. Usually, the cassette may be prepared underneath a small tank filled with transfer buffer solution. The transfer usually takes place for 1 hour at 100 volts in the former while about 15-20 mins at 20 volts in the later. After the transfer is finished, the success of the transfer was visualized by a reversible staining with a ponceau-S solution. The blot was stained into a ponceau-S solution for 10 min and then was washed with water to remove unbound stain. The desired region of the blot is next marked and cut out or

alternatively the whole gel may also be used in certain cases. Later blot was unstained using 1-X TBST for 10 min on slow shaking. The membrane was next blocked using 5% non-fat dry milk in TBST solution. It was then incubated with a primary antibody solution at 4°C overnight on a shaker. The next day, the excess antibody solution was washed off using TBST 5 times for 5 min each wash. The blot was further incubated with an HRP conjugated secondary antibody solution (diluted in 5% milk) for 1h shaking at room temperature. Excess secondary antibody was again washed off using TBST. 1-2 ml of substrate solution was spread over the membrane and was incubated for 1 min. Either X-ray film was exposed to the blot and further developed or fluorescence scanner was used to detect bands on the membrane.

2.2.14 CD spectroscopy

Steady state circular dichroism (CD) was measured in the far UV light (180-260 nm) using a CHIRASCAN spectropolarimeter (Applied Photophysics). Spectra were collected in a 60 ul quartz cell (Hellma) at 20°C at a step resolution of 1 nm. CD spectrum of recombinant proteins purified was recorded in different buffers to study the effect of buffers on the secondary structure of the same. The spectrum of the buffer was automatically subtracted from the spectra of the protein using the software provided by the manufacturer. The baseline corrected spectra was used as an input for computer methods to obtain predictions of secondary structure (Bohm, Muhr et al. 1992).

2.2.15 Nuclear magnetic resonance (NMR) spectroscopy

2.2.15.1 Isotopic Labelling of recombinant proteins

All recombinant proteins, labelled with ^{15}N , were purified for 2D NMR experiments. Human FKBP25 was also labeled with ^{13}C or both ^{15}N and ^{13}C for 3D NMR experiments. Genes encoding the protein were previously cloned into SUMO vectors or pET29b and transformed into BL21 (DE3) *E. coli* cells. Transformed BL21 (DE3) cells were inoculated into 10 ml M9 media containing ^{15}N labelled ammonium chloride. List of reagents for M9 media is given below.

Table 2.6 Components for M9 medium

For ^{15}N labelling	For ^{13}C labelling	For $^{15}\text{N}/^{13}\text{C}$ labelling
52.7 mM Na_2HPO_4	52.7 mM Na_2HPO_4	52.7 mM Na_2HPO_4
26.5 mM KH_2PO_4	26.5 mM KH_2PO_4	26.5 mM KH_2PO_4
10 mM NaCl	10 mM NaCl	10 mM NaCl
0.1 mM CaCl_2	0.1 mM CaCl_2	0.1 mM CaCl_2
1.2 mM MgSO_4	1.2 mM MgSO_4	1.2 mM MgSO_4
4.5 g/l Glucose	2 g/l ^{13}C Glucose	2 g/l ^{13}C Glucose
5 ng/l Thiamine	5 ng/l Thiamine	5 ng/l Thiamine
1 g/l $^{15}\text{NH}_4\text{Cl}$	1 g/l NH_4Cl	1 g/l $^{15}\text{NH}_4\text{Cl}$
Antibiotic	Antibiotic	Antibiotic

The overnight grown culture was subcultured into 1 litre M9 media with appropriately labelled reagent and antibiotic. The culture was grown till OD reached 0.6 followed by IPTG induction at 25°C for 6-8 h shaking. Cells were harvested and the labelled FKBP25 and deletion constructs were then purified following the same protocol used to purify unlabelled FKBP25. The NMR samples were prepared in 20 mM phosphate, 50 mM NaCl and 0.01% NaN_3 . 0.1 mM protein was used for 1D and 2D NMR experiments while for 3D experiments 0.5 mM protein was used. All protein samples were prepared in 10% D_2O for all 2D and 3D NMR experiments.

2.2.15.2 Data collection and NMR experiments for backbone and side chain assignment

The resonance assignments were achieved using 2D and 3D heteronuclear NMR experiments, performed on uniformly ^{15}N and $^{13}\text{C}/^{15}\text{N}$ -labeled samples. All NMR experiments were carried out on a Bruker Avance 600 MHz spectrometer equipped with a cryoprobe at 298 K. ^1H - ^{15}N HSQC, HNCACB, HNCA, HN(CO)CA, CBCA(CO)NH, HNCO and HN(CA)CO experiments were performed in order to complete the backbone assignment. Side chain assignments were obtained from the spectra of (H)CC(CO)NH, H[CC(CO)] NH, ^{13}C -HSQC-NOESY, ^{15}N -HSQC-NOESY (Satller et al. 1999, Simon et

al.2004). Aromatic ring resonance was assigned from ^{13}C -NOESY-HSQC, ^{15}N -NOESY-HSQC. The acquisition mode used was states/TPPI (time proportional phase incrementation). All data were processed with NMRPipe (Delaglio et al. 1995) and spectra were analyzed using SPARKY (Goddard et al. 2002).

2.2.15.3 Backbone and side chain assignments

By several 2D and 3D NMR experiments, one can assign resonance of all residues of labeled protein. HNCACB, HNCA, HN(CO)CA, CBCA(CO)NH, HNCO and HN(CA)CO experiments were used for backbone assignment which includes resonance of $\text{C}\alpha$, $\text{C}\beta$, CO, N and N-H. HNCACB spectrum is used to assign $\text{C}\alpha$ and $\text{C}\beta$ of the i^{th} and $i-1^{\text{th}}$ amino acid residue. Peaks of i^{th} residues are normally stronger than that of $i-1^{\text{th}}$ residue. The spectrum of CBCA(CO)NH gives information about $\text{C}\alpha$ and $\text{C}\beta$ of $i-1^{\text{th}}$ residue. Another important spectrum is HNCA spectrum, which deals with peaks for $\text{C}\alpha$ of the i^{th} and $i-1^{\text{th}}$ residues. HN(CO)CA spectrum shows peaks for $\text{C}\alpha$ of $i-1^{\text{th}}$ residue. HN(CA)CO spectra are used to assign carbonyl carbon of each amino acid as each peak represents resonance of C of the i^{th} residue. Similarly, HNCO gives information of the resonance of C of the i^{th} and i^{th} residue. Thus employing all of the above 3D NMR experiments, the backbone assignment was completed.

After finishing the backbone assignment, side chain residues (^1H and ^{13}C) were assigned. HNHA was performed to assign chemical shift of all $\text{H}\alpha$. (H)CC(CO)NH was used for side chain ^{13}C assignment. The (H)CC(CO)NH spectrum gives information about $\text{C}\alpha$, $\text{C}\beta$, $\text{C}\gamma$, $\text{C}\delta$ and $\text{C}\epsilon$ of $i-1^{\text{th}}$ amino acid. H[CC(CO)] NH experiment gives information about all ^1H of the side chain of $i-1^{\text{th}}$ amino acid. ^{15}N -TOCSY was also used for ^1H assignment as this experiment tells us about the resonance of all ^1H of the side chain of $i-1^{\text{th}}$ amino acid. Further, ^{15}N -NOESY and ^{13}C -NOESY were carried out to assign resonance of ^1H and ^{13}C of the side chain.

The ^1H resonances of free DNA^{YY1} oligomer were assigned using a combination of homonuclear 2D TOCSY and NOESY (τ_m 200 ms) recorded in 90% H_2O /10% D_2O or 99.9% D_2O conditions. DNA assignments in the FKBP25-DNA^{YY1} complex were obtained from 2D-F1, F2- $^{13}\text{C}/^{15}\text{N}$ -filtered NOESY spectra (Breeze, 2000; Iwahara et al., 2001; Ogura

et al., 1996; Zwahlen et al., 1997). Intermolecular NOEs between FKBP25 and DNA^{YY1} were assigned from 3D-¹³C F1-filtered F3-edited NOESY spectra recorded in D₂O (Breeze, 2000; Iwahara et al., 2001; Ogura et al., 1996; Zwahlen et al., 1997). All spectra were processed with an NMRPipe (Delaglio et al., 1995b) and analyzed using SPARKY. The assigned chemical shift values of FKBP25 have been deposited in the Biological Magnetic Resonance Bank (accession code 16738). To measure residual dipolar coupling (RDC) constants, poly(ethylene glycol)/alcohol mixtures were used as alignment media for the preparation of anisotropic sample condition as described previously (Rückert and Otting, 2000). Briefly, 50 µL of C12E5 were mixed in 530 µL of NMR buffer containing 90% H₂O and 10% D₂O for stock solution preparation. 1-Hexanol was gradually added in 2µL increments with vigorous shaking to a final molar C12E5: 1-hexanol ratio of 0.96. After 1 h of resting at room temperature, air bubbles were removed by centrifugation at 5000×g for several minutes. For the measurement of RDC, 300 µL of the C12E5:1-hexanol stock solution was added to 200 µL of protein solution. The final concentration of the C12E5:1-hexanol mixture in the NMR sample was about 5% (wt/wt). One-bond N-NH RDC constants were measured using 2D ¹H-coupled IPAP ¹H-¹⁵N-HSQC spectra (Cordier et al., 1999) with 512 complex t₁ (¹⁵N) points and 128 scans per t₁ increment for both isotropic and anisotropic conditions. The data analysis and calculation of the alignment tensor were performed using REDCAT software (Valafar and Prestegard, 2004)

2.2.15.4 Structure calculation and refinement of human FKBP25

The solution structures of the human FKBP25 were calculated in two step process. Firstly, we performed automatic structure calculation by simulated annealing in torsion angle space with a combination of the programs CYANA 2.1(Guntert, 2009) and CNS 1.2 (Brunger et al., 1998). After getting initial structures, we manually assigned Nuclear Overhauser Effect (NOE) distance constraints which were derived by analyzing ¹H-¹⁵N-NOESY-HSQC (100 ms mixing time) and ¹³C-edited NOESY-HSQC (100 ms mixing time) spectra of uniformly ¹⁵N- or ¹³C/¹⁵N-labeled samples of FKBP25. We performed second round of structure calculation with the input of manually assigned NOEs, other constraints like dihedral angle and Hydrogen bonds. The ambiguous peaks were manually assigned based on the initial structures. The secondary structure was predicted by the TALOS+

program (Shen et al., 2009) based on the results of the analysis of chemical shifts of the main-chain N, HA, CA and C atoms and sequential ($|i-j|=1$), short range ($|i-j|<5$) NH-NH and NH-aliphatic contacts on a ^1H - ^{15}N -NOESY-HSQC spectrum. Dihedral angle (ϕ , ψ) restraints were also calculated from chemical shifts using TALOS+ and hydrogen bond restraints were obtained based on the protein structure during structure calculations. NOE cross-peaks on NOESY spectra were classified based on their intensities and were applied with an upper distance limit of 3.0 Å (strong), 3.5 Å (medium), 5.0 Å (weak) and 6.0 Å (very weak), respectively. An additional 0.5 Å was added for NOEs that involved methylene and methyl groups. Upper distance bounds for the inter-domain NOE contacts between the N (HLH) and C (FKBD) domains were set at 6.5 Å. A total of 200 conformers were generated as initial structures by CYANA 2.1 from 5,836 NOE and 281 backbone dihedral angle constraints. After calculation, initial conformers were sorted by target function values and the lowest 100 conformers were selected for further refinement using CNS 1.2. One hundred and thirty-six backbone hydrogen bonds were identified on the basis of initial structures and 128 ^1DNH RDC constraints were included in the final stage of the calculation. The final structure was refined using a simulated annealing protocol with a combination of torsion angle space and cartesian coordinate dynamics as described previously (Brunger, 2007). Finally, 20 structures were selected by their total energy values for display and structural analysis. MOLMOL (Koradi et al., 1996) and PyMOL (DeLano, 2009) programs were used for structure visualization and PROCHECK-NMR and Protein Structure Validation Software suite were used for structure validation (Bhattacharya et al., 2007; Laskowski et al., 1996a). The 20 NMR ensemble structures have been deposited in the Protein Data Bank with code 2MPH.

2.2.15.5 Paramagnetic relaxation enhancement (PRE) experiment

To observe the intermolecular interaction between FKBP25 and DNA, oligonucleotides with dT-EDTA at positions 4 and 20 were purchased from Sigma-Aldrich (Singapore). The oligonucleotides were annealed with their respective unlabeled strand to generate two types of dsDNA denoted as DNA-1 (labeled at dT4) and DNA-2 (labeled at dT20) (Figure 6c). The dT-EDTA-labeled dsDNAs were mixed with equal amounts of Mn^{2+} or Ca^{2+} to achieve a paramagnetic or diamagnetic state, respectively, and free Mn^{2+}

or Ca^{2+} was subsequently removed on a PD-10 column. For PRE measurements, NMR samples (0.15 mM) were prepared by mixing ^{15}N -labeled FKBP25 with a DNA- Mn^{2+} or DNA- Ca^{2+} complex in a 1:1 molar ratio. ^{15}N TROSY-HSQC spectra were acquired on a Bruker 600 MHz NMR spectrometer equipped with a cryoprobe. The peak intensities of paramagnetic and diamagnetic states were measured and the intensity ratios of the paramagnetic to diamagnetic state ($I_{\text{par}}/I_{\text{dia}}$) were calculated.

2.2.15.6 NMR titration of FKBP25 with DNA oligomer or rapamycin

Molecular interaction between FKBP25 and DNA or rapamycin were studied using 2D TROSY-HSQC spectra of ^{15}N -labeled FKBP25 by the addition of DNA oligomer recorded on a Bruker Avance 700 spectrometer at 298 K. NMR samples were prepared in 25 mM Tris, pH 7, and 150 mM NaCl buffer with 10% D_2O . Initially, the NMR spectrum of the free sample was recorded using 0.1 mM of ^{15}N -labeled FKBP25. The DNA oligomers were then added to the protein sample at molar ratio 1 (FKBP25) to 1 (DNA) and 1 to 2. In case of rapamycin titration, 2 μl of rapamycin from the 50 mM stock solution (dissolved in DMSO), was added slowly into 500 μl of FKBP25 (10 % D_2O). After comparison of changes of the chemical shifts before and after of the DNA or rapamycin, the weighted chemical shift perturbations for backbone ^{15}N and ^1HN were calculated by the formula $\Delta d = [(\Delta d\text{N}/5)^2 + (\Delta d\text{HN})^2]^{0.5}$ and DNA interaction sites were mapped on the protein structure.

2.2.16 HADDOCK docking

With the inputs of NMR titration, intermolecular interactions between DNA and FKBP25 derived from isotope filtered NOESY experiment and mutagenesis data, we performed docking on a HADDOCK web server as previously described (de Vries et al., 2010) and built a model for the FKBP25-DNA complex. As the sequence of first 20 residues of 23-bp DNA^{YY1} used in this study were same as the sequence of DNA 20-bp dsDNA used in the crystal structure of YY1-DNA complex (PDB code 1UBD), we used the structure of DNA from the YY1-DNA complex and the solution structure of the full-length human FKBP25 for docking experiments. The active residues of FKBP25 were defined by combinations as those showing chemical shift perturbations larger than the

averages ($\delta > 0.05$), the presence of intermolecular NOEs between FKBP25 and DNA and mutagenesis data with relative residue accessible surface area larger than 30 % for either main chain or side chain atoms by calculated with NACCESS and MOLMOL program. The active residues of DNA were defined from 3D- ^{13}C F1-filtered F3-edited NOESY data. Based on NMR titration, isotope filtered NOESY, and mutagenesis data, we defined Lys22, Lys23, Lys42, Lys48, Gln150, Lys156 and Lys157 as active residues for protein. Passive residues were automatically picked by the HADDOCK server program. In the case of DNA, based on ambiguous or unambiguous intermolecular NOE information, we selected nucleotides A10, C23, T24, T25, C26, G32, A34 and G35 as active residues. These experimental restraints were used as input for the HADDOCK program and default parameters were used for docking. The resulting structures were clustered by a default cutoff value (7.5Å). HADDOCK clustered 153 structures in 10 clusters, which represent 76.5 % of the water-refined models HADDOCK generated. Finally, based on the HADDOCK score and total energy, the best model from the first cluster was selected as the final model of the FKBP25-DNA. The quality of the HADDOCK-derived complex models was checked using PROCHECK (Laskowski et al., 1996b).

2.2.17 DNA gel retardation assay

Plasmid DNA (pSUMO) was transfected into competent DH5 α cells and left to grow overnight at 37°C in an incubator. The plasmid DNA was then extracted using a plasmid extraction kit from Qiagen. A total of 300 ng of either supercoiled or linearized pSUMO plasmid or pGEX-4t plasmid were mixed with FKBP25 protein at different FKBP25/DNA molar ratios (0, 25, 125, 250 and 500) in 10 μl of 20 mM phosphate buffer and 150 mM NaCl. The protein-DNA mixture was incubated at room temperature for 30 min and loaded on 1% agarose gel. To make a Linearized DNA, the plasmid was digested by *EcoRI*. Single-stranded pSUMO was geared up by heating the linearized plasmid to 95°C for 5 min then quenching it to 0°C.

In order to obtain Single-stranded DNA, linearized plasmid was heated at 95°C for 5 min and then subjected to fast cooling. To investigate whether DNA-protein interaction is primarily mediated by electrostatic interaction, FKBP25-DNA mixture was prepared with different NaCl concentration ranging from 0 mM to 1600 mM. The plasmid –FKBP25

mixture was incubated for 30 min at room temperature at a molar ratio 1:250 before loading in 1% agarose gel in TBE buffer. Gel was electrophoresed at 65 V for 1.5 h and ethidium bromide was used for visualization of the bands.

We used 1% agarose gel to verify interaction of wild-type and mutant FKBP25 with DNA. Wild-type and mutant FKBP25 proteins were incubated with 300 ng of pSUMO plasmid for 1h at room temperature. After incubation, protein-DNA complex was loaded on 1% agarose gel and run at 60 V and stained by EtBr for visualization.

2.2.18 Isothermal titration calorimetry (ITC) experiment

The binding of a 23-bp double-stranded deoxyoligonucleotide (DNA^{YY1}) with FKBP25 and YY1-DBD, and also FKBP25 with YY1-DBD were analyzed by ITC experiments carried out on a MicroCal iTC200 (MicroCal Inc., Northampton) at 25 °C. For the FKBP25-DNA^{YY1} binding study, 100 μ M 23-bp DNA^{YY1} was titrated into 25 μ M FKBP25 or into the ITC buffer containing 25 mM Tris and 150 mM NaCl at pH 7.0. For each titration, 1 μ L of DNA was injected 24 times with a time interval of 180 s and the stirring speed was maintained at 500 rpm. The reference power was 7 μ cal s⁻¹. For YY1-DBD and DNA^{YY1} interaction, 200 μ M dsDNA^{YY1} was titrated into 25 μ M YY1-DBD by mixing 2.5 μ L of DNA per injection for 16 injections. In order to study FKBP25 and YY1-DBD interaction, 0.7 mM FKBP25 was titrated into 50 μ M YY1-DBD. A total of 19 injections were made with 2 μ l per injection. For both YY1-DBD bindings with FKBP25 or DNA^{YY1}, the time interval for each injection was 150 s and the stirring speed was fixed to 500 rpm. For all ITC experiments involving YY1-DBD, all proteins and DNA samples were prepared by dialyzing samples into aliquots of stock buffer containing 25 mM Bis-Tris, pH 7.0, 150 mM NaCl and 0.1 mM ZnCl₂ to avoid any signal obtained from buffer mismatch. For the buffer blank experiments, DNA or FKBP25 was titrated into the same buffer. The data collected from all of the ITC experiments were integrated using MicroCal Origin 5.0 and signals from blank were subtracted. The data was fitted to a one-site binding model (due to mixing artifacts, the heat associated with the first peak was excluded from the data analysis).

2.2.19 Tryptophan quenching experiment

To confirm the binding of 23-bp DNA^{YY1} with FKBP25 and its mutants, change in the intrinsic fluorescence of the tryptophan moieties of FKBP25 were recorded using a Cary Eclipse fluorescence spectrophotometer (Varian, Inc.). A total of 5 μ M of human FKBP25, K22A, Q150E or K157A was titrated with DNA^{YY1} and fluorescence intensity was recorded until the binding reached to saturation. The sample was excited at 290 nm and emission spectra were recorded between 300 nm and 420 nm at 25 °C. Relative fluorescence intensity, which was obtained by $[(F_0-F)/F_0]$ where F_0 and F are the fluorescence intensities in absence and presence of DNA, was plotted against the DNA concentrations. The binding affinity K_d was determined by Origin pro software using ligand depletion model.

2.2.20 Screening for protein crystal

Crystallization screenings were done with different screening buffers from different commercial manufacturers. Recombinant pure protein was concentrated up to different concentrations *e.g.* 5 mg/ml, 10 mg/ml, 15 mg/ml and then used for crystallization set up. The solution was initially centrifuged at 12000 rpm for 10 minutes to remove any dust particles or aggregates and the supernatant was used for the experiment. 1 μ l of protein solution is mixed with 1 μ l of buffer and set up for crystal growth using hanging drop method. The drops were next analyzed at regular intervals for the appearance of any growth under a microscope.

2.2.21 Crystallization and X-ray diffraction experiments

Crystallization screen was performed using the hanging-drop vapor diffusion method, with FKBD25 at 12 mg/mL mixed with FK506 at a molar ratio of 1:2 and incubated overnight at 4 °C. Equal volumes of the protein and reservoir solutions were mixed and sealed with 500 μ l of reservoir solution in each well. Crystals of FKBD25-FK506 complex appeared in 0.1 M HEPES pH7.0 and 30 % v/v Jeffamine ED-2001 pH 7 after 4-5 weeks. The crystals were cryoprotected with 20 % glycerol added to reservoir solution for data collection at 100 K on beamline 13B1 at the National Synchrotron Radiation Research Center (Hsinchu, Taiwan) using an ADSC Q315 detector.

2.2.22 Structure determination by X-ray crystallography

The data was indexed, integrated, merged and scaled using the software iMosflm (Battye et al., 2011) and SCALA (Evans, 2006) from CCP4 suite of programs (Winn et al., 2011). The crystal belonged to the trigonal space group $P3_2 2 1$, with one molecule in the asymmetric unit. The initial phases were obtained by molecular replacement calculated using PHASER (McCoy et al., 2007) and the protein atoms from the FKBD25-rapamycin complex (PDB ID 1PBK) (Liang et al., 1996) were used as the model. REFMAC (Murshudov et al., 2011) and COOT (Emsley and Cowtan, 2004) were used for refinement and map fitting respectively while PyMOL (DeLano, 2002) was used to generate the figures. The electron density for the FK506 atoms could be identified unambiguously at the active site. Additional electron density could be observed at the C-terminal end corresponding to residues Leu225 and Glu226, resulting from a cloning artifact, followed by two His residues of the 6X His tag. Water molecules were manually picked from the Fo-Fc and 2Fo-Fc electron density map contoured at 3.0 and 1.0 σ cut-offs, respectively. In addition, a part of the jeffamine (Ligand Id: 6JZ) could be identified near the active site, while another one, with a few missing atoms, could be located near the $\beta 5$ - $\beta 6$ loop. The crystallization condition has been the source of this Jeffamine. The FKBD25-FK506 interactions were identified using LigPlot (Wallace et al., 1995) and manual inspection while the structure based sequence alignment was performed by PROMALS3D (Pei and Grishin, 2014) and EsPript (Gouet et al., 2003). The coordinates and structure factors of the FKBD25-FK506 complex have been deposited in the Protein Data Bank (PDB ID).

Chapter 3A

Cloning, Expression and purification of FKBP25

3.A Cloning, expression and purification of HLH domain, FKBD and full length FKBP25.

3.A.1 Aim and overview of study

FKBPs are known to bind with FK506 or rapamycin with their well conserved FK506 binding domain. The binding affinity of rapamycin or FK506 to the canonical FKBP12 is almost the same, but FKBP25 shows a relatively lower binding affinity to FK506 in comparison to rapamycin. In fact, the binding affinity of rapamycin ($K_i = 0.9$ nM) to FKBP25 is comparable to other FKBPs, unlike the binding affinity of FK506 ($K_i = 200$ nM) which is almost 200 fold low. The molecular basis underlying the lower binding affinity of FK506 to FKBP25 remains elusive. Though the crystal structure of the FKBD25 (FK506 binding domain of FKBP25) in complex with rapamycin has been solved long ago (Liang et al., 1996), its FK506 complex is not available till date. Hence, the aim of this study is to understand the molecular basis for the differential binding affinity of FKBP25 with FK506 and rapamycin. Towards this end, we cloned, expressed, purified and solved the crystal structure of human FKBD25 in complex with FK506 and compared it with its rapamycin counterpart.

Immunophilins are well characterized to have multi-domains in their full length structure, yet there are very few reports that observe interactions between these domains. Therefore, very little is understood about how these domain-domain interactions contribute to protein functionality. One such rare study characterized the domain-domain interaction in Pin1, another member of the immunophilin family, thereby illustrating the significance of domain-domain interaction to study their behaviour. As FKBP25 bears two distinctly different domains which are linked through a long flexible loop, it would be interesting to solve the structure of full-length FKBP25 and investigate the dynamics of any possible domain-domain interaction. Another interesting observation that paves way for further scrutiny is that previous studies have indicated that FKBP25 can bind DNA. In order to further understand how FKBP25 binds with DNA, which domain/residues of FKBP25 are important for such binding, how the topology of these two domains is important for DNA binding, it was imperative to solve the structure of full-length FKBP25. Towards this end, we cloned, expressed, purified and solved the solution structure of FKBP25 by NMR.

Overall in this study, we attempted to achieve three main goals which are as follows. (1) Clone, express and purify full-length FKBP25 and its individual domains. (2) Solve crystal structure of FKBD of FKBP25 in complex with FK506 drug. (3) Solve the solution structure of full-length FKBP25.

3.2 Cloning, expression, and purification of full-length FKBP25 and its deletion mutants

Human FKBP25 was cloned in the pSUMO expression vector. The SUMO expression vector produces protein tagged with pSUMO and 6xHis tag (Figure 3.1C). Because of the SUMO fusion, the solubility and yield of the recombinant protein increase. Gene encoding human FKBP25 was amplified by PCR using appropriate primers (see section 2.2.1) and the amplicon was run on a 1 % agarose gel. The figure 3.1A shows PCR amplification of 675 bp of FKBP25 gene. Both the amplified gene product and pSUMO vector was digested with *BsaI* and *BamHI* and digested products were ligated by T4 DNA ligase. Ligated products were transformed into DH5 α cells and plated on LB agar plate containing kanamycin (30 μ g / ml). 10 colonies were randomly picked and used for colony PCR as mentioned in section 2.2.3. Among ten colonies, seven colonies were positive clones as shown by PCR amplification (Figure 3.1B). Both positive controls had amplification while both negative controls showed no amplification, indicating that the positive clones were true clones. For further confirmation, two of the clones were sent for sequencing and the sequencing result showed that both of the clones were correct in sequence and we used clone1 for further study.

Figure 3.1 PCR amplification of FKBP25 gene (A) 1 % agarose gel showing PCR amplification of FKBP25 gene. Lane 1 is 100bp marker and lane 2 is the PCR amplicon while lane 3 is the non-template control. (B) The result of colony PCR. Lane 1 is a marker, lanes 2-11 are clones, lane 12-13 are positive controls and lane 14-15 are negative controls. It is observed that 7 out of 10 clones were positive clones. Positive controls have amplification while negative controls do not show any amplification. (C) The plasmid map of the pSUMO-FKBP25 vector.

We also required different deletion constructs for several experiments to characterize the structure and function of FKBP25. These constructs are (1) N-terminal HLH domain of FKBP25 represented as FKBP25(1-90 aa), (2) N-terminal HLH domain with the flexible loop of FKBP25 represented as FKBP25(1-109 aa) and (3) C-terminal FK506 binding domain represented as FKBD25. The protocol for cloning and expression of all the deletion constructs was identical to that of full-length FKBP25, except for the FKBD25 where the SUMO tag could not be digested by SUMO protease. So FKBD25 was cloned again in pET29b vector using a new set of primers.

For the expression of full-length FKBP25, FKBP25(1-90aa) and FKBP25(1-109aa) as SUMO fusion proteins, all transformed cells (BL21 *E.coli*. strain) were induced by 0.2 mM IPTG. Figure 3.2 shows that FKBP25 (also Figure 3.4A) and its deletion constructs FKBP25(1-90aa) and FKBP25(1-109aa) were expressed with a good yield (15-25 mg/ml).

Figure 3.2 Induction and solubility test of full-length FKBP25, FKBP25(1-90) and FKBP(1-109). Gel picture shows full-length FKBP25 (A), FKBP25(1-90) (B) and FKBP25(1-109) (B) proteins got expressed as SUMO fusion proteins. Cells were induced with 0.2 mM IPTG for 4h at 25°C. Cell lysate before and after IPTG induction are labeled as (-) or (+) respectively. SUMO:FKBP25(C), SUMO:FKBP25(1-90aa) (C) and SUMO:FKBP25(1-109aa) (C) proteins were present in the supernatant fraction of cell lysate, indicating that these proteins are soluble and well folded in lysis buffer. Supernatant and pellet fraction are labelled as ‘S’ and ‘P’ respectively.

On the other hand, FKBD25 (without SUMO tag, as it was cloned in pET29b), was also induced with 0.2 mM IPTG (Figure 3.3). Once we ascertained that FKBP25 and its deletion constructs were expressed, we tested the solubility of these proteins in lysis buffer. Soluble protein was found in the supernatant fraction of lysed cells while insoluble protein precipitates into pellet fractions. For that purpose, 4 h IPTG-induced cells were lysed by sonication and centrifuged to collect supernatant and pellet fraction. Both supernatant and pellet fraction were then run on 12 % SDS-PAGE. FKBP25 and its deletion constructs were soluble in the buffer, as we could find these proteins in supernatant fractions (Figure 3.2C, D; Figure 3.3 and Figure 3.4A).

Figure 3.3 Induction and solubility test of FKBD25. Gel picture shows that FKBD25 was both induced and soluble indicated by its presence in the supernatant fraction of induced cell lysate. Cells were induced with 0.5 mM IPTG for 4h at 25°C. Cell lysate before and after IPTG induction are labeled as (-) or (+) respectively. (B) Induced cells were lysed and centrifuged. Lanes loaded with the supernatant and pellet are indicated as 'S' and 'P' respectively.

After confirming that the proteins were soluble, we optimized their purification. All proteins were purified by Ni-NTA column and the collected fractions were run on SDS-PAGE for analysis. For purification of proteins, two rounds of Ni affinity chromatography were carried out, except for FKBD25 (as it was not expressed as a SUMO fusion protein), in order to get rid of the SUMO tag. Samples collected after the first round of purification had the SUMO-His tag (Figure 3.4A). To remove the SUMO tag, these fusion proteins were digested with the SUMO protease enzyme. Digested fusion proteins were subjected to the second round of purification (Figure 3.4B). The SUMO along with His tag gets trapped into the column as it binds with greater affinity to Ni-NTA beads, thereby allowing pure untagged proteins to be eluted in the flow-through fraction. FKBD25 was not cloned in SUMO vector, so it did not bear the SUMO tag and got purified in the first round of purification. Figure 3.2 B and D show that the purified FKBP25 and its deletion constructs were pure enough to be used for further studies. To confirm that the purified protein was FKBP25, we performed western blotting of the SUMO:FKBP25 using an antibody against His-tag (Figure 3.4 C).

Figure 3.4 purification of FKBP25 and its individual domains (A) Gel picture showing expression and purification of human FKBP25. Human FKBP25 was expressed in the *E.coli* BL21 strain. Protein was purified by Ni-NTA column and different fractions were analyzed by SDS-PAGE. ‘Induced’ denotes cell lysate from cells induced with IPTG. (B) SUMO:FKBP25 fusion protein was purified in the first round of purification, followed by sumo protease digestion to remove the SUMO tag. Digested product was used for the second round of purification to get untagged FKBP25. Purified FKBP25 without any tag was collected in flow through fraction. FKBP25 (1-90aa) and FKBP25 (1-109aa) were also purified similarly as full-length FKBP25. (C) Western blotting confirming that purified protein was recombinant FKBP25. (D) Purified FKBP25 (1-90aa), FKBP25 (1-109aa) and FKBD25 (from left to right).

3.3 Biophysical characterization of FKBP25

3.3.1 Size exclusion chromatography

Gel filtration is one of the most popular methods for measuring the size of a protein and the estimated molecular weight gives a clue about the oligomeric state of the protein. In order to investigate whether FKBP25 makes any oligomer or not, we performed gel filtration on Superdex-200 column. Analysis of gel filtration data showed that a homogeneous population of FKBP25 was eluted at 83.54 ml elution volume (Figure 3.5 A).

Figure 3.5 Gel filtration of FKBP25 showing the monomeric state of FKBP25 (A) Protein was loaded onto superdex-200 column to check the homogeneity of the protein. FKBP25 was eluted at 83.54 ml. (B) Estimation of molecular weight from a standard plot. Estimated molecular weight is 24 kDa which is comparable to the expected molecular weight (25.2 kDa) and thus indicates that FKBP25 is a monomer in solution.

Using the molecular weight standard curve; the molecular weight of FKBP25 was estimated to be 24 kDa, comparable with its known molecular weight 25.2 kDa (Figure 3.5 B) which indicates that FKBP25 exists as a monomer.

3.3.2 1D and 2D NMR experiments

After purifying a protein, the first step toward solving the structure of the protein is to confirm its proper folding. NMR is an important tool for such studies. 1D and 2D NMR experiments were done to obtain primary information about protein folding. For 1D NMR experiments 0.1 mM purified FKBP25 was used. Peaks in 1D NMR spectrum were observed to be well dispersed which confirms that the protein is well folded and present in a globular form (Figure 3.6A). Good dispersion of resonance line of methyl proton (-0.5-1.5 ppm), α -proton (3.5-6 ppm) and amide proton (6-10 ppm) was observed.

^{15}N HSQC spectrum gives unique peaks for all amino acids except proline. As mentioned in the methods section, uniformly ^{15}N labeled FKBP25 sample was prepared for 2D ^1H - ^{15}N HSQC experiment. The HSQC spectra showed well-dispersed peaks of backbone amide which confirms that FKBP25 was properly folded (Figure 3.6B). There was some crowding in the spectrum because of the dynamic loop, connecting two domains of FKBP25. Both 1D and 2D spectrum indicated that purified FKBP25 is folded correctly and can be used for backbone and side chain assignments. Later we also checked the folding of HLH and FKBD by collecting and analyzing HSQC spectra of respective proteins. Figure 3.7 shows that both of the HLH and FKBD were well folded.

Figure 3.6 Results of NMR experiments showing a well-folded state of purified FKBP25. (A) 1D ^1H NMR spectrum of purified FKBP25. Peaks are well dispersed showing protein in folded state. Methyl H appeared between 0 to -1 ppm which further indicates a correctly folded state of the protein. (B) 2D ^1H - ^{15}N HSQC spectrum of FKBP25. Spectrum was collected on 600 MHz spectrometer at 298K. The protein sample was prepared in 20 mM phosphate buffer pH7, 50 mM NaCl, and 10 % D_2O . The spectrum shows the good dispersion of backbone amides except a few overlapped peaks at the center.

Figure 3.7 Results of NMR experiments showing a well-folded state of purified (A) FKBD25 and (B) HLH domain of FKBP25. The spectrum shows the good dispersion of backbone amides. Spectrum was collected on 600 MHz spectrometer equipped with cryoprobe at 298K. The protein samples were prepared in 20 mM phosphate buffer pH7, 50 mM NaCl, and 10 % D_2O .

Chapter 3B

Crystal structure of FKBD25 in complex with the FK506 drug

3B: Crystal structure of FKBD25 in complex with the FK506 drug

In order to understand the differential binding affinity FKBP25 to its inhibitors, we have solved the crystal structure of FKBD25 in complex with FK506.

3B.1 Structure determination of FKBD25-FK506 complex

C-terminal FKBD (residues 109-224) of FKBP25 (referred as FKBD25), was purified as mentioned in the Materials and Methods section. Folding of FKBD25 was monitored by 2D NMR HSQC spectra (Figure 3.7 A). In order to obtain a crystal and solve the structure of FKBD25 in complex with FK506, we screened several crystal screening conditions. Crystallization screen was performed using 12 mg / mL FKBD25 mixed with FK506 at 1:2 molar ratio. The crystals of FKBD25-FK506 complex appeared in 0.1 M HEPES pH 7.0 and 30 % v/v Jeffamine ED-2001 after 4-5 weeks. These crystals were tested in the in-house machine first and good diffraction quality crystals were measured at the National Synchrotron Radiation Research Center (NSRRC, Hsinchu, Taiwan). The crystal of FKBD25-FK506 complex diffracted to a maximum resolution of 1.8 Å resolution. The crystal belonged to $P 3_2 2 1$ space group, with unit-cell parameters $a = 74.78$ Å, $b = 74.78$ Å, $c = 44.62$ Å. The crystal structure at resolution 1.8 Å resolution enabled us to unambiguously trace all FK506 atoms in the electron density map (Figure 3.8). The summary of data collection and processing statistics of the FKBD25-FK506 complex are summarized in Table 3.1.

Figure 3.8: The 2Fo-Fc electron density map of FK506 in complex with FKBD25, contoured at 1σ cut-off. The FK506 molecule is shown in stick mode.

Table 3.1 X-ray data and refinement statistics for the FKBD25-FK506 complex crystal

Data Collection	
Wavelength (Å)	1.000
Space Group	P 3 ₂ 2 1
Unit Cell Parameters	
a ; b ; c (Å)	74.78 ; 74.78 ; 44.62
α ; β ; γ (°)	90.00 ; 90.00 ; 120.00
Resolution (Å)	30.00 – 1.83 (1.90-1.83) [†]
R _{merge}	0.045 (0.556)
Unique Reflections	12952 (1264)
Mean [(I)/ σ (I)]	34.4 (2.0)
Completeness	99.7 (98.3)
Multiplicity	5.5 (3.2)
Refinement	
Number of Reflections	11605
Resolution (Å)	25.00 – 1.83
R-Value	0.1870
R-Free	0.2331
No. of atoms	
Total / Protein / FK506 / Hetero / Water	1146 / 949 / 57 / 34 / 106
Mean B-Value (Å ²)	
Total / Protein / FK506 / Hetero / Water	25.41 / 23.16 / 22.56 / 54.37 / 37.75
R.m.s.d. from ideal values	
Bond Lengths (Å)	0.011
Bond Angles (°)	1.549
Torsion Angles (°)	7.079
Ramachandran Statistics (%)	
Preferred Regions	95.0
Allowed Regions	5.0
Outliers	0.0

[†] indicates values at the highest resolution

3B.2 Structure of FKBD25 in FKBD-FK506 complex

The overall structure of FKBD25 in FKBD25-FK506 complex is very similar to the previously reported FKBD25 in the FKBD25-rapamycin complex. As expected, the crystal structure showed that FK506 binds to the canonical hydrophobic pocket of FKBD25 (Figure 3.9). Like all other FKBDs of human FKBP, FKBD25 also consists of five antiparallel β -strands which make curved β -sheets and also a short α helix (Figure 3.10). The hydrophobic pocket of FKBD25 consists of well-conserved hydrophobic residues like Y135, F145, V171, W175, I208 and F216 which correspond to Y27, F37, V56, W60 and F100 respectively of FKBP12, the simplest member of the FKBP family which shows almost 43 % sequence similarity with FKBD25. Mainly β 4, β 5, short α -helix, 40s loop, 50s loop and 80s loop contribute to FK506 binding pocket formation. The r.m.s deviation (RMSD) of FKBD25-FK506 complex is 0.59 Å for 74 equivalent α -carbon atoms and 0.45 Å for 101 equivalent α -carbon atoms with the FKBP12-FK506 and FKBD25-rapamycin structures respectively, which indicates that the protein atoms in these complexes adopt almost similar conformations.

Figure 3.9: Structure of FKBD25 in complex with FK506. (A) The surface model of FKBD25 from the complex showing the hydrophobic pocket which accommodates FK506 or rapamycin. (B) The structure of FKBD25 bound with FK506 has been shown in a different angle. FK506 drug binds and fits into the hydrophobic pocket of FKBD25.

3B.3 Interactions of FK506 with FKBD25

Similar to rapamycin, the pipercolinyl ring of FK506 deeply penetrates into the hydrophobic pocket. In the hydrophobic pocket, pipercolinyl ring of FK506 gets surrounded by residues like Y135, L162, V171, I172, W175 and F216. The pyranose ring of FKBD25 shows hydrophobic interaction with residues like A206, I208, F145, Y198, and D146. Similar to FKBP12-FK506 complex, FK506 also makes four hydrogen bonds with residues D146, K170, I172 and Y198 of FKBD25 (Figure 3.11). The piperconyl amide carbonyl group at C8 and ester carbonyl group at C1 accept a hydrogen bond from Y198 and I172 respectively. C10 hydroxyl makes a hydrogen bond with D146-O^δ while C24 hydroxyl makes hydrogen bonds with K170-O. Overall, these conserved hydrogen bonds bridge the three ends of the FK506 (O2/O10; O3 & O6) molecule with the residues (Ile172/Lys170; Tyr198 & Asp146) and the pipercolyl moiety forming the fourth end (base of FK506) is mainly stabilized by non-bonded interactions with several hydrophobic residues. All four hydrogen bonds and all the hydrophobic interactions have been summarized in Table 3.2 and figure 3.11. The bond length of all four hydrogen bonds and residues involved in hydrophobic interaction has been shown in Figure 3.11.

Figure 3.10 Comparison of crystal structure FKBD25 bound either with FK506 or rapamycin. (A) The cartoon diagram of the FKBD25-FK506 complex with FK506 represented as sticks. The structure shows that FKBD25 consist of typical FKBD fold with 6 β strands and 1 α helix. All the β strands, loops, and α helix has been labeled accordingly. (B) The superimposed structure of FKBD25-FK506 complex and FKBD25-rapamycin complex (PDB - 1PBK). It is very obvious that both FK506 and rapamycin bind almost in the same fashion. There are not many structural changes in FKBD25 when it is bound with either of FK506 or rapamycin.

Table 3.2: The interactions made by FK506 or rapamycin with FKBD25 or FKBP12

Hydrogen bonds								
FKBD25-FK506			FKBD25-Rapamycin			FKBP12-FK506		
FK506 Atom	FKBD25 Atom	Distance(Å)	Rapamycin Atom	FKBD25 Atom	Distance(Å)	FK506 Atom	FKBP12 Atom	Distance (Å)
O2	Ile172 N	2.8	O2	Ile172 N	2.9	O2	Ile56 N	2.8
O3	Tyr198OH	2.8	O3	Tyr198 OH	2.7	O3	Tyr82 OH	2.8
O6	Asp146OD2	2.6	O6	Asp146 OD2	2.7	O6	Asp37 OD2	2.8
O10	Lys170 O	2.6	O10	Lys170 O	2.7	O10	Glu54 O	2.7
			O8	Lys170 NZ	3.0			
			O9	Lys170 NZ	3.0			
			O13	Gly169 O	2.9			
Non-bonded Contacts								
FKBD25-FK506			FKBD25-Rapamycin			FKBD12-FK506		

FK506 Atom	FKBD25 Residues	Rapamycin Atom	FKBD25 Residues	FK506 Atom	FKBP12 Residues
C3	Trp175	C3	Trp175	C3	Trp59
C4	Leu162, Trp175	C4	Leu162, Trp175	C4	Phe46, Val55, Trp59
C5	Tyr135, Leu162, Trp175	C5	Tyr135, Leu162	C12	His87
C6	Tyr135	C43	Ile208	C35	Ile91
C12	Ala206			C36	Tyr26, Phe46
C35	Ala206, Ile208			C41	Phe46
C36	Leu162				
C43	Gln203				
C45	Tyr198				
C-H...O Interactions					
FKBD25-FK506		FKBD25-Rapamycin		FKBD12-FK506	
FK506 Atom	hFKBD25 Residues	Rapamycin Atom	hFKBD25 Residues	FK506 Atom	hFKBP12 Residues
O2	Val171, Ile172	O2	Val171, Ile172	O2	Val55, Ile56
O3	Tyr198, Phe216	O3	Tyr198, Phe216	O3	Phe99
O4	Tyr135, Phe145, Asp146, Phe216	O4	Tyr135, Phe145, Phe216	O4	Tyr26, Phe36, Phe99
O6	Asp146	O8	Lys170	O6	Asp37
		O10	Lys170		
		O11	Val171		
C6	Tyr135	C2	Tyr198	C6	Tyr26
C11	Tyr198	C11	Tyr198	C11	Tyr82
C12	Gln203	C35	Tyr198	C12	His87
C26	Lys170	C37	Lys170	C15	Tyr26, Asp37
C28	Lys170	C39	Gly169, Lys170	C36	Tyr26
C42	Tyr198	C49	Tyr198	C41	Glu54
C43	Gln203	C52	Gly169	C42	Tyr82
C45	Ala197			C45	Ala81

Figure 3.11 Interaction of FKBD25 with FK506 drug. (A) A 2D interaction map of FKBD25-FK506 complex generated by LIGPLOT (Wishart, 1994). All atoms of FK506 are labeled. This interaction map shows all four residues, which forms hydrogen bonds with FK506, as ball and stick. All the other residues shown are those that form hydrophobic interactions with FK506. (B) Hydrogen bonds and hydrophobic interactions made by FK506 with FKBD25. FK506 (in green color) and active site residues of FKBD25 are also shown as thin and thick sticks respectively. The residues making hydrogen bond (along with their distances) are shown in gray-white; those forming non-bonded interactions are shown pale yellow colors.

3B.4 Comparison of FKBD25-FK506 complex structure with the structures of FKBD25-rapamycin and FKBP12-FK506 complexes

In order to understand the lower affinity of rapamycin toward FKBP25 ($K_i = 0.9$ nM) in comparison to FKBP12 ($K_i = 0.26$ nM), we have compared the sequence FKBD25 with FKBP12 and also the structure of FKBP25-FK506 with both FKBP25-rapamycin and FKBP12-FK506 complex. Firstly, we compared all similar and dissimilar residues of FKBD25 with FKBP12 and then looked into the changes in the structural features due to changes in these residues. In FKBP12, a total of fourteen residues (Tyr26, Phe36, Asp37, Arg42, Phe46, Glu54, Val55, Ile56, Trp59, Tyr82, His87, Ile90, Ile91 and Phe99) show direct contacts (less than 4 Å) with FK506 which correspond to Tyr135, Phe145, Asp146, Asn158, Leu162, Lys170, Val171, Ile172, Trp175, Tyr198, Gln203, Ala206, Ile207 and Phe216 in FKBP25. Among all above residues, dissimilar residues in FKBP12/FKBD25 are Arg42/Asn158, Phe46/Leu162, Glu54/Lys170, His87/Gln203, Ile90/Ala206 while others are similar residues (Figure 3.12).

Figure 3.12 Sequence alignment of human FKBD25 with human FKBP12. Below the sequences, identical active site residues have been marked with asterisks while non-identical residues with crossed square box.

After picking these dissimilar residues, we grouped them based on which part of protein they belong to and then try to understand that how these change could lead to the different selectivity of FKBP25 for its inhibitors. These dissimilar residues mainly belong to 40s loop (Arg42/Asn158), 50s loop (Glu54/Lys170), 80s loop (His87/Gln203 and

Ile90/Ala206) and $\beta 4$ (Phe46/Leu162). One after one, we have looked into structural changes in above mentioned regions of FKBP25.

(1) Changes in 40s loop: In the FKBP12 active site, Asp37 forms a salt bridge with Arg42 and hydrogen bond with Tyr26-OH while Arg42 also form a hydrogen bond with Tyr26-OH and thus they form an Arg-Asp-Tyr triad which could be important for FK506 binding (Van Duyne et al., 1993). In FKBP25, this salt bridge is lost due to the substitution of Arg42 by Asn158, while the other two residues are conserved (Tyr26 to Tyr135 & Asp37 to Asp146) (Figure 3.13). In addition to this, the Asn158 moves away from the other two residues due to the extension of the 40s loop in FKBP25 as the 40s loop of FKBD25 is uniquely long. The loss of this triad could be one of the reasons for low affinity of FK506 to FKBP25 with respect to FKBP12.

Figure 3.13 The comparison of the 40s loop of FKBD25 and FKBP12. The triad formation by Try26, Asp37 and Arg42 residues (shown in cyan colored sticks) of FKBP12 (in the green color cartoon). Although Tyr135 and Asp146 (blue sticks) are present in the same position in FKBD25 (in the purple color cartoon), corresponding to Tyr26 and Asp37 in FKBP12, Asn158 (red sticks) is very far from other two residues and cannot form tirad.

(2) Changes in 80s loop: In the hydrophobic pocket of FKBP12, residues from 80s loop like Y82, H87, I90 and I91 interacts with pyranose ring of FK506. Among these residues His87/Gln203 and Ile90/Ala206 has been substituted while others are conserved. Although Ile90 of FKBP12 is substituted with Ala206, this substitution could not affect binding as Ala206 also adopts a similar conformation as Ile90 to make hydrophobic interaction with pyranose ring of FK506. In the case of a substitution of His87 from Gln203, the interaction

between Gln203 and pyranose is abolished. Thus, this substitution could reduce binding of FKBP25 to both of its inhibitors FK506 and rapamycin.

Figure 3.14 Comparison of FKBD25 with FKBP12 in complex with FK506 complexes. (A) Cartoon representation of the superposition of the FK506 complexes of FKBD25 (blue) and FKBP12 (pale brown), with the active site residues shown in stick mode and FK506 molecules in ball and stick mode. A closer view of the identical (B) and non-identical residues (C) are also shown for clarity along with their respective numbering.

(3) Change in residue Phe46 to Leu162 from β 4 strand: Another important difference between FKBP12 and FKBD25 is the substitution of the conserved Phe46 by Leu162 (Figure 3.14C). Phe46 of FKBP12 is substituted with Leu162 and both of them can contribute to hydrophobic interaction, but leucine provides a smaller van der Waals surface than phenylalanine. So Phe66 to Lue162 substitution could adversely affect the hydrophobic packing and interactions in the region. Thus, this substitution could lead to reduced binding affinity of FKBP25 with both rapamycin and FK506 with respect to FKBP12.

(4) Changes in 50s loop: When we observed closely the residues of 50s loop, we realized that the residue Lys170 could provide some important clues. In FKBP25 negatively charged Glu54 is substituted by positively charged residue (Lys170). In FKBP12, Glu54 forms one hydrogen bond both with FK506 and rapamycin. Same as FKBP12, FKBP25 also forms one hydrogen bond with both rapamycin and FK506 by the C28 and C24 hydroxyl respectively. Interestingly, the side chain nitrogen atom (NZ) of Lys170 makes two additional hydrogen bonds with C26 carbonyl and C27 methoxy oxygen atoms of rapamycin (Figure 3.15 B, Table 3.2). In comparison with rapamycin, FK506 lacks these atoms which are involved in hydrogen bond formations resulting in the absence of this additional two hydrogen bonds, despite the fact that Lys170 adopts similar orientation in both these structures (Figure 3.4.15 B). In addition, a part of jeffamine, from the crystallization condition, observed between the FK506 molecule and Lys170, probably compensates for the interactions made by rapamycin. A superposition of the FK506 and rapamycin complexes of FKBP12 revealed that the side chain oxygen of Glu54 forms hydrogen bonds neither with FK506 nor with rapamycin, which emphasizes the importance of the two additional hydrogen bonds made exclusively by Lys170 to rapamycin but not FK506. Apart from the above differences, the backbone oxygen of the neighboring residue Gly169 also forms a hydrogen bond with O13 of the cyclohexyl ring of rapamycin, but not with FK506 (Figure 3.4.15B, Table 3.2). One reason could be due to the flip of the cyclohexyl ring of FK506 (by $\sim 77^\circ$ in comparison to that of rapamycin), away from Gly169 eliminating the possibility of a hydrogen bond. A similar difference in the cyclohexyl ring orientation could also be observed between the rapamycin and FK506

bound structures of FKBP12. Incidentally, in the case of FKBP12 a larger amino acid (Gln53) replaces the Gly169, probably counterbalancing for the loss of the hydrogen bonding.

Figure 3.15: Comparison of the interactions made by the residues Tyr135, Asp146, Leu162, Gly169 and Lys170 of FKBP25 with FK506 (light green ball and stick mode) and Rapamycin (white ball and stick mode). (A) The interactions made by the residues Tyr135, Asp146 and Leu162 with FK506 and rapamycin, indicating fewer differences. (B) On the contrary, the hydrogen bonds formed by Lys170 and Gly169 with rapamycin are lost in the FK506 complex. It could be observed that the conformations of these two residues are similar in both these complexes, implying that the lack of donor atoms in FK506 at this site could be responsible for its reduced affinity in comparison to rapamycin.

In conclusion, the substituted residues of FKBD25 like Gln203 and Leu162 may have an adverse effect on binding of both of the FK506 and rapamycin in comparison to FKBP12. Further, the substitution of Glu54 to Lys170 could compensate for the decrease in binding affinity by providing two additional hydrogen bonds for rapamycin but not for FK506 and thus making rapamycin a stronger binder to FKBP25 with respect FK506. Mutagenesis of Lys170 followed by affinity characterization remains to be done to substantiate our observation and it will be performed in our lab in future.

This study also shows that how loss and gain of the binding property of FKBP25 for rapamycin make the binding affinity of rapamycin to FKBP25 comparable with FKBP12. Also, the loss of only some hydrophobic interactions makes the binding affinity of FKBP25 with FK506 weaker than rapamycin. In conclusion, the structure of FKBD25 in complex with FK506 and their comparisons presented here has helped us unravel the probable molecular basis for its lower affinity toward FKBP25 compared to rapamycin.

Chapter 3C

Solution structure of full-length FKBP25

3C: Solution structure of full-length FKBP25

In order to understand the topology of two domains of FKBP25, we have solved the solution structure of full-length FKBP25. There are three main steps in the structure determination of a moderately large protein by NMR (1) backbone assignment (2) side chain assignment and (3) structure calculation and refinement.

3C.1 Backbone assignments of FKBP25

Backbone assignment includes resonance assignment of ^{15}N , HN, $^{13}\text{C}_\alpha$, $^{13}\text{C}_\beta$ and ^{13}C . These resonance assignments were completed by the information collected from HNCACB, HNCA, CBCA(CO)NH, HN(CO)CA, HNCO, HN(CA)CO triple resonance experiments (for detail see Materials and Methods). Uniformly labelled FKBP25 protein sample were prepared in phosphate buffer and 10 % D_2O . NMR experiments were carried out in 600 MHz NMR spectrometer (Bruker, Switzerland) at 298K. All spectra were processed by NMRPipe (Delaglio et al., 1995a) and analyzed by SPARKY (Goddard.).

Using above mentioned NMR experiments, we were able to obtain near complete assignments for the backbone and side chain resonances of the full-length FKBP25 protein. ^1H and ^{15}N backbone chemical shifts of 96.87 % of the non-proline residues of FKBP25 were assigned.

Figure 3.16 Strip plot obtained from HNCA spectrum showing sequential connectivity of C_α from residues K110 to K118. Each strip has two peaks; the one which is stronger in intensity represents C_α of i^{th} amino acid while the weaker represents C_α of $i-1^{\text{th}}$ amino acid. The green line in the figure shows sequential connectivity.

Among 213 non-proline amino acid residues of FKBP25, amide peaks for 7 non-proline residues (M1, A2, K56, K110, T151, A153 and K200) were missing and ^1H and ^{15}N chemical shifts all other residues were assigned. ^{13}C chemical shifts were assigned for 213 non-proline residues by achieving almost 98.6 % completeness. 212 out of 213 C_α , 198 out of 199 C_β and 206 out of 213 CO were assigned. By combining triple resonance and ^{13}C -edited NOESY spectra, we were also able to almost fully assign the C_α , C_β and CO resonances of 11 proline residues. Due to sequential proline–proline sequence, we were unable to assign CO chemical shifts of P108 and P209 residues. Strip plotting was done to

check connectivity among amide resonance. Strip-plot showed that peaks were well connected and correctly assigned (Figure 3.16). The fully assigned HSQC spectrum of FKBP25 is shown in the Figure 3.17 and side chain amides are shown by horizontal lines in the Figure 3.17.

Figure 3.17: Backbone assigned 2D ^1H - ^{15}N HSQC spectrum of FKBP25. ^1H and ^{15}N resonance were assigned using information from triple resonance experiments like HNCACB, HNCA, CBCA(CO)NH, HN(CO)CA, HNCO, HN(CA)CO. All experiments were carried out using 600 MHz spectrometer equipped with a cryoprobe at 298K. 0.5 mM protein sample was prepared in 20 mM phosphate buffer pH7, 50 mM NaCl and 10% D_2O . All amides are labeled with the residue sequence number and one-letter amino acid code. Signals from side chain amide of Asn and Gln are joined by a horizontal line. Resonance peaks with asterisk show indolic nitrogen and proton resonances of tryptophan. The overlapped region is zoomed and shown in a separate box.

3C.2 Side chain assignment of FKBP25

Side chain resonances of all side chains ^1H (H_α , H_β , H_γ , H_δ and H_ϵ) were assigned by HNHA, HCC(CO)NH and HCCH-TOCSY spectra and all ^{13}C (C_α , C_β , C_γ , C_δ , and C_ϵ) resonances were assigned by CCONH spectra. Almost 97.6 % of all non-labile aliphatic ^1H (223/224 of H_α , 209/210 of H_β and 302/306 of other side-chain ^1H) and ^{13}C resonances (223/224 of C_α , 209/210 of C_β , 267/296 of other aliphatic side-chain ^{13}C) and 95.2 % of ^1H resonances of all aromatic side chain (59/62) were assigned. Further, all ^1H and ^{13}C resonances were assigned and confirmed by ^{15}N -NOESY and ^{13}C -NOESY respectively. In addition, the assignment of side-chain labile ^1H and ^{15}N resonances of 10 Asp, 7 Glu, 2 Arg and 4 Tyr were completed among the 10 Asp, 8 Glu, 5 Arg and 4 Tyr residues.

Figure 3.18 Predicted secondary structure of FKBP25 plotted using the consensus chemical shift index (CSI). $^1\text{H}_\alpha$, $^{13}\text{C}_\alpha$, $^{13}\text{C}_\beta$ and ^{13}CO chemical shifts were used to get this plot. The values of CSI for α -helix (represented by rectangular box), β -strand (represented by arrow) and random coil are -1, +1 and 0 respectively. Secondary structure prediction suggested that N-terminal domain has five α -helices while C-terminal domain has six β -strands and one α -helix.

Prediction of the secondary structures was performed using CSI (Wishart and Sykes, 1994) and TALOS+ Program (Shen et al. 2009), and ^1HN - ^1HN or ^1HN - H_α NOE patterns based on the 3D- ^{15}N -edited NOESY-HSQC spectrum. Figure 3.18 shows the secondary structural elements estimated by calculation of CSI program. The chemical shift deviation from random coil values of all H_α , $^{13}\text{C}_\alpha$, $^{13}\text{C}_\beta$ and ^{13}CO has been plotted for all residues of FKBP25 in Appendix 2. The estimated secondary structure of FKBP25 showed helix-loop-

helix domain fold on the N-terminal side (1–73), long random coiled linker on the middle of the sequence (74–108) and typical FK506 binding protein fold similar to other FKBP s on the C-terminal side (109–224). The chemical shift data has been deposited in the BioMagResBank (<http://www.bmrb.wisc.edu>) under accession number 19551. The assigned chemical shift data could be further used for structural and protein/ligand interaction studies of FKBP25 protein.

3C.3 Solution structure of full-length human FKBP25

Structure calculation and refinement was performed by Shin Joon, a postdoctoral fellow from our lab. Using the backbone and side-chain assignments, determined the solution structure of the full-length human FKBP25. A total of 5,972 NMR-derived distance restraints, 281 dihedral angle restraints, and 128 RDC constraints were included in the final step of structure calculation. The theoretical RDC value estimated after structure calculation and the experimental RDC values were well matched. The ensemble of 20 low-energy structures, calculated with CNS is shown in Figure 3.19A. Excluding the 35 residues (Gly74 - Pro108) in the flexible loop between the N-terminal HLH and C-terminal FKBD, the root-mean-square deviation (RMSD) values relative to the mean coordinate of 20 conformers were 0.67 Å for the backbone atoms and 1.01 Å for all heavy atoms (Table 3.3). The topology of FKBP25 showed that the N-terminal HLH consists of five α -helices (α 1: 12-16, α 2: 23-32, α 3: 35-40, α 4: 47-51 and α 5: 56-69). The C-terminal FK506 binding domain (FKBD) shows similar structure as FKBD25 in complex with either FK506 or rapamycin. Similar to the FKBDs of all other human FKBP s, FKBD25 consists of six beta strands and one short alpha helix labeled as α 6. These two domains are connected by an unstructured and relatively long flexible linker from Gly74-Pro108 (Figure 3.19A). The electrostatic potential surface revealed that there are a number of positively charged residues on both HLH domain and FKBD which suggests that FKBP25 may bind to nucleic acids. The possibility of nucleic acid binding by FKBP25 was further explored and studied in detail (Chapter 4). The calculated RMSD values for the C_{α} trace between previously reported HLH domain and HLH of our NMR structure was 1.40 Å (Figure 3.20A). Although the structure of free FKBD25 was not solved before, the calculated RMSD values for the C_{α} trace between FKBD of our NMR structure and the

FKBD from the FKBD25-rapamycin complex is 1.87 Å (Figure 3.20B). The reason for higher RMSD is the binding of rapamycin would have brought some structural changes in FKBD.

Table 3.3: Structural statistics for FKBP25

Number of NOE constraints	
All	5835
Intra residues $ i-j =0$	1008
Sequential, $ i-j =1$	1568
Medium-range, $1< i-j <5$	1011
Long-range, $ i-j \geq 5$	2248
Number of Hydrogen Bond Constraints	136
Number of Dihedral Angle Constraints	281
Number of RDC Constraints ($^1D_{\text{HN}}$)	128
Number of Constraint Violations ($>0.5\text{\AA}$)	0
Number of Angle Violations ($>5^\circ$)	0
Number of RDC Violations ($>1.0\text{Hz}$)	0
CNS energy (kcal.mol^{-1})	
E_{total}	133.58 ± 1.58
E_{NOE}	6.73 ± 0.53
E_{cdih}	0.62 ± 0.14
$E_{\text{bond}} + E_{\text{angle}} + E_{\text{improper}}$	91.86 ± 1.40
E_{vdw}	33.29 ± 1.41
RMSD for Residue 2-73,109-223 to mean (excluding flexible loop 74-108) ^a	
Backbone	$0.67 \pm 0.25\text{\AA}$
Heavy Atoms	$1.01 \pm 0.26\text{\AA}$
RMSD for N-terminal helix-loop-helix domain (2-73) ^a	
Backbone	$0.55 \pm 0.13\text{\AA}$
Heavy Atoms	$0.86 \pm 0.16\text{\AA}$
RMSD for C-terminal FK506 binding domain (109-223) ^a	
Backbone	$0.60 \pm 0.32\text{\AA}$
Heavy Atoms	$1.00 \pm 0.34\text{\AA}$
Ramachandran Plot (excluding flexible loop 74-108) ^b	
Most Favored Regions	83.1%
Additionally Allowed Regions	14.5%
Generously Allowed Regions	2.4%
Disallowed Regions	0.0%

Figure 3.19: NMR solution structures of the FKBP25 (A) A superposition of the backbone traces from the final ensembles of 20 solution structures of the full-length FKBP25 determined by NMR spectroscopy (left image). The N-terminal HLH (residue M1-K73) and C-terminal FKBD (residue P109-D224) are highlighted in red and blue respectively. A ribbon representation of FKBP25 is displayed, using the same color scheme, to indicate the domains, and the central flexible loop (G74-P108) linking the two domains is highlighted in green (right image). The uniquely long basic loop of FKBD (shown in circle) is present in close vicinity to HLH domain. (B) Ensembles of 20 solution structures (left), a ribbon representation (middle) and the electrostatic potential surface (right) of the N-terminal HLH and C-terminal FKBD of FKBP25 are displayed for brevity. The secondary structural elements are labeled as indicated. The positively charged surface is shown in blue and the negatively charged surface is in red.

Figure 3.20: Superposition of each domain of FKBP25 on the previously reported structures. (A) Ribbon representation of the overlay of the HLH domain between our NMR structure (PDB 2KMP, red) and previously reported NMR structure (PDB 2KFV, blue). (B) Ribbon representation of the overlay of the FK506 binding domain between our NMR structure (magenta) and previously reported crystal structure (PDB 1PBK, green).

3C.4 Domain-domain interaction of FKBP25

For several proteins, interdomain- domain interaction has been reported and such interactions have been suggested to be important for the full functional activity of the protein. One of the members of the immunophilin family, Pin1 exhibits inter-domain communication between WW domain and PPIase domain and this interaction was found to be important for its PPIase activity. Interestingly, the structure of FKBP25 also showed that there is an inter-domain cross talk between HLH domain and FKBD which is a unique feature of FKBP25 among all other FKBP. The N-terminal HLH domain (Val5-Arg8) interacts with residues of the C-terminal $\beta 2$ and $\beta 6$. From the ^{15}N -edited NOESY-HSQC and ^{13}C -edited NOESY-HSQC spectra, we could observe few inter-domain NOEs between N-terminal HLH and C-terminal FKBD (Figure 3.21A and B). Those NOE were long-range and weak NOEs. No NOEs could be observed that should be present based on the structure as they were missing.). The symmetric NOE of Q7 on the carbon plane of K187 has been shown in Appendix 3. There were hydrogen bond formation between the backbone amides of Val5 and Gln7 and the side chain oxygen atoms of the Thr138 and the

Glu217, respectively (Figure 3.21C). In addition, we could also observe an ionic interaction between Arg8 and Glu219 (Figure 3.21C).

Figure 3.21: Molecular interaction between the N-terminal HLH and C-terminal FKBD domains. (A) Representative strips of the ^{15}N -edited three-dimensional NOESY spectrum of FKBP25 showing NOE cross-peaks between residues on the N-terminal (Gln7) and the C-terminal FKBD (Tyr135) (B) representative strips of the ^{13}C -edited three-dimensional NOESY spectrum of FKBP25, with NOE cross-peaks between residues on the N-terminal HLH (Pro6, Gln7) and the C-terminal FKBD (Phe216, Lys187) (C) A ribbon representation of the interaction between the HLH domain and FKBD. The HLH domain and FKBD are highlighted in pink and cyan respectively. Crucial residues forming hydrogen bonds (Val5, Gln7, Thr138 and Glu217) and electrostatic interactions (Arg8, Glu219) are shown as sticks and hydrogen bonds are indicated by dashed lines.

To study internal motion and the overall domain dynamics, we measured ^{15}N relaxation data for FKBP25 (Appendix 4). R1, R2 and heteronuclear data corroborate that FKBP25 comprises of well-ordered N- terminal HLH and C-terminal FKBD connected by a flexible linker. The average tumbling correlation time for overall residues in the N-terminal HLH domain is $c \approx 7.5$ ns, which is significantly larger than the estimated value of $c \approx 5.9$ ns, using HYDRONMR software (Garcia de la Torre et al., 2000). For C-terminal FKBD, the average c of 11 ns is slightly higher than the theoretical value, $c \approx 10$ ns. These values suggest that the rotational diffusion of both domains are coupled and do not tumble independently. However ^{15}N R2/R1 ratios and different correlation times of the HLH and FKBD are significantly different, indicating that the interaction between both domains is not strong. These results suggest that HLH and FKBD of FKBP25 are in between the two extreme dynamics models. The first model is single rigid tumbling of both domains, and the second one is each two domain is dynamically independent. ^{15}N relaxation data and observation of weak interdomain NOEs support FKBP25's interdomain flexibility and weak domain–domain interaction between both HLH domain and FKBD.

For a multi-domain protein which shows inter-domain interactions, the binding of a drug to one domain could bring some structural changes in the other domain, because of inter-domain interaction. Thus, binding of a drug to one domain of a protein and structural change in another domain of the same protein could be used as an indication of domain-domain interaction. We know that FK506 or rapamycin binds to a C-terminal domain of FKBP25, but whether this binding could have some effect on N-terminal HLH was unknown. We performed NMR titration of FKBP25 in presence or absence of rapamycin and as expected, the overlaid spectra showed notable chemical shift perturbations (CSPs) in the FKBD. Interestingly, some residues from HLH domain also showed CSPs upon rapamycin binding (Figure 3.22). . The CSP in N-terminal HLH upon rapamycin binding to the C-terminal FKBD is only possible when there is a domain-domain interaction between HLH and FKBD.

Figure 3.22: Chemical shift perturbations of N-terminal HLH domain of FKBP25 upon addition of rapamycin. (A) The plot of chemical shift perturbations of FKBP25 upon binding of rapamycin at 1:2 molar ratio of FKBP25 to rapamycin. The differences of chemical shifts were calculated using the following formula, $\Delta\delta = [({}^1\text{H}_{\text{free}} - {}^1\text{H}_{\text{bound}})^2 + ({}^{15}\text{N}_{\text{free}} - {}^{15}\text{N}_{\text{bound}})^2]^{1/2}$. Chemical shift perturbation of N-terminal HLH domain has been enlarged and residues affected by the addition of rapamycin are labeled with a *single letter*. (B) Representative expanded sections of overlaid ^1H - ^{15}N HSQC spectra show the significant chemical shift perturbations on some residues on N-terminal HLH. NMR titration experiments were performed on a Bruker Avance 600 spectrometer at 298K, using uniformly ^{15}N -labeled FKBP25 (red) and FKBP25 in the presence of rapamycin (blue) at a molar ratio of 1 (FKBP25) to 2 (rapamycin). The perturbations of chemical shifts are displayed by arrows, and perturbed residues are labeled with a three letter on top of the sections.

To further confirm domain-domain interaction, we purified the HLH domain of FKBP25 and collected the HSQC spectra. The overlay of FKBP25 with HLH domain caused only slight perturbations of the chemical shifts on the N-terminal HLH domain. It indicates the truncation of FKBD from FKBP25 caused a slight change in the conformation of HLH domain which indirectly indicates the domain-domain interaction between the N-terminal HLH and C-terminal FKBD (Figure 3.23). Although we could not observe a relatively high chemical shift for the residues like Gln7 and Val 5, we could observe a slight change in the chemical shifts of several residues. This data suggests the presence of weak domain-domain interaction, which supports our model of FKBP25 in which there is an interdomain flexibility as well as a weak domain–domain interaction.

The connecting loop between the N- and C-terminal domains is unstructured in solution as demonstrated by the lack of medium and long-range NOEs and also random coil secondary chemical shifts and dynamic properties determined in a heteronuclear NOE experiment. These data suggest that the HLH and FKBD cooperate with each other to perform their molecular functions.

Further to confirm the presence of domain domain interaction, we will would like mutate residues which shows NOE between two domains and then study the domain domain interaction. In future we will also perform PRE experiments by protein spin labels to support the model architecture of FKBP25.

Figure 3.23: Chemical shift perturbations of N-terminal HLH of FKBP25 caused by deletion of C-terminal FKBD. (A) The plot of chemical shift perturbations of the N-terminal domain of FKBP25 upon deletion of C-terminal FKBD. The difference of chemical shifts was calculated using the following formula, $\Delta\delta = [({}^1\text{H}_{\text{HLH}} - {}^1\text{H}_{\text{full-length}})^2 + ({}^{15}\text{N}_{\text{HLH}} - {}^{15}\text{N}_{\text{full-length}})^2]^{1/2}$. The residues affected by deletion of C-terminal FKBD are labeled with a single letter. (B) Representative expanded sections of overlay of ${}^1\text{H}$ - ${}^{15}\text{N}$ HSQC spectra of uniformly ${}^{15}\text{N}$ -labeled full-length FKBP25 (red) and HLH domain. The perturbations of chemical shifts from full-length FKBP25 to HLH domain (blue) are displayed by arrows, and perturbed residues are labeled with a three letter on top of the sections.

Chapter 4

Characterization of nucleic acid binding properties of FKBP25

4.1 Aim and overview of study

In general, most nuclear proteins associate with one or several form of nucleic acid (RNA, double stranded DNA, single stranded DNA, quadruplex DNA) either in a sequence specific or sequence independent manner. Proteins that recognize DNA in a sequence-dependent manner are mainly associated with transcription regulation of the specific gene. On the contrary, proteins which bind to DNA in sequence independent manner are involved in DNA bending, nucleosome assembly, DNA chaperone activity, DNA repair etc. FKBP25, a unique member of the FKBP family due to its localization in the nucleus, is a relatively less characterized protein. It binds with some of the important nuclear proteins like HMG (Leclercq et al., 2000), nucleolin (Jin and Burakoff, 1993; Wishart et al., 1994b), MDM2 (Ochocka et al., 2009), HDAC (Hua et al., 2003) and YY1 (Yang et al., 2001). Previous studies suggest that FKBP25 can bind DNA (Riviere et al., 1993). The aim of this study is to understand the mechanism of binding of FKBP25 with different nucleic acids. Towards this end, we have shown that FKBP25 binds with DNA in a sequence-independent manner. This binding was confirmed by several biophysical methods like NMR, gel shift assay, ITC and fluorescence spectroscopy, supported by mutational studies. We extended our studies and mapped the DNA binding site on FKBP25 and proposed a model for the FKBP25-DNA complex.

4.2 Evidence for FKBP25 DNA binding

In order to investigate any possible role of FKBP25 in nucleic acid recognition, we mapped the electrostatic potential surface of the N-terminal HLH (Figure 4.1A) and C-terminal FKBD of FKBP25 (Figure 4.1B). The mapping revealed that several lysine residues made up a positively charged surface on the N-terminal HLH domain. The residues responsible for this charged surface are K22, K23, K27, K42, K48 and K52. We also observed a similar charged surface on FKBD, made by residues K113, K118, K121, K154, K155, K156, K157 and K213. Based on these observations, we proposed a possible role of FKBP25 in DNA recognition and performed several experiments to characterize FKBP25-DNA interaction.

Figure 4.1: The electrostatic potential map of the two individual domains of FKBP25 (A) HLH domain and (B) FKBD. The positively charged residues (labelled in yellow color) have been represented in blue color. The structural characteristics of the full-length FKBP25 revealed that it is a potential nucleic acid-binding protein.

4.3 Human FKBP25 binds to double-stranded plasmid DNA in a sequence-independent manner

To investigate the binding of FKBP25 with DNA, we performed gel shift assay. Two different supercoiled plasmids (pSUMO and pGEX-4T) were purified and used as DNA source for the gel retardation assay. In principle, if a protein binds to DNA and forms a protein-DNA complex, the size of complex becomes bigger than the free DNA which leads to slower migration of the complex with respect to free DNA. Gel shift assay using plasmid DNA has been used before to study the DNA binding property of Tau (Hua et al., 2003) and several other proteins which bind DNA in sequence independent manner. As we suspected that FKBP25 could bind DNA, we performed gel shift assay with pSUMO in the presence of FKBP25 protein. pSUMO DNA was mixed with increasing concentrations of FKBP25 and the protein-DNA mixture was loaded onto 1 % agarose gel. The result showed that there was a gradual decrease in the migration of the pSUMO as we increase the concentration of FKBP25 (Figure 4.2A). The retardation was clearly detectable when the molar ratio was close to 1:125. We obtained similar results with pGEX-4T plasmid (Figure

4.2B). The fact that FKBP25 could bind to both, pSUMO and pGEX-4T (which has an entirely different sequence), and also that a high molar ratio of FKBP25 was required to bind with DNA, suggests that FKBP25 binds DNA in sequence non-specific manner. The observation that increase in the concentration of FKBP25 resulted in increased retardation in gel shift can be explained by the fact that size of the plasmid is bigger than FKBP25, so one molecule of plasmid DNA could bind to several molecules of FKBP25. Thus, as we increase the concentration of FKBP25, the number of FKBP25 bound to each plasmid will increase which in turn increases the size of complex and thus more retardation could be observed as we increase protein concentration in gel shift assay. In order to further confirm this sequence non-specific interaction, we incubated FKBP25 with 100 bp DNA ladder and ran them on a 1 % agarose gel (Figure 4.2C). All DNA bands of the 100 bp DNA ladder showed a significant shift upon FKBP25 binding, which further confirms that FKBP25 interacts with DNA with no sequence specificity.

Figure 4.2: Gel mobility shift experiments showing binding of FKBP25 with DNA. A gradual increase in band shift in purified plasmids (A) pSUMO and (B) pGEX-4T 300 ng each were mixed with increasing concentrations of FKBP25 (molar ratio 1:0, 1:1, 1:25 and 1:125; lane 1-4 respectively) is shown. (C) The retardation of DNA fragment migration after FKBP25 binding is shown; DNA fragments of all sizes (ranging from 3 kb to 100 bp) display reduced migration.

4.4 Human FKBP25 does not bind to single-stranded DNA

Some DNA binding proteins bind both double-stranded (dsDNA) and single-stranded DNA (ssDNA) while others recognize either only dsDNA or ssDNA. After confirming the binding of double-stranded DNA (dsDNA) with FKBP25, we were prompted to test whether FKBP25 can also bind to single-stranded DNA (ssDNA). We prepared ssDNA by digesting pSUMO with *BamHI* restriction enzyme, followed by heating at 95 °C for 10 min and then snap cooling. The result of gel shift assay showed that there was no retardation in the mobility of ssDNA in the presence of FKBP25 (Figure 4.3A). We also confirmed this observation by performing NMR experiments which have been discussed in section 4.5.1. These findings suggest that FKBP25 has a preferred DNA binding to dsDNA over ssDNA. This indicates that though FKBP25 recognizes dsDNA in a sequence-independent manner, it is unable to bind to ssDNA implying that FKBP25 might need some structural features specific to dsDNA for its recognition.

4.5 Interaction of FKBP25 with dsDNA is salt dependent.

In general, the sequence-independent DNA binding is mostly mediated by ionic interactions between basic amino acids of the protein and the phosphate backbone of DNA. Thus, an increase in salt concentration causes a decrease in the binding affinity of the protein to DNA. As gel shift assay suggested that FKBP25-DNA binding is also a sequence-independent binding (Figure 4.2), we investigated the effect of salt concentration of FKBP25-DNA binding. We performed gel shift assay for FKBP25 and pSUMO DNA mixture with increasing concentrations of NaCl. The result showed that when we added no salt, there was a maximum shift for FKBP25-DNA complex with respect to free DNA. When we gradually increased salt concentration from 0 mM to 1600 mM of NaCl, we could see a gradual increase in mobility (or decrease in the retardation of the DNA migration) (Figure 4.3B). At a salt concentration of 1600 mM, the shift in the band was same as free DNA which indicated that this salt concentration was able to completely abolish the FKBP25-DNA interaction. Further, we also confirmed that FKBP25-DNA interaction is salt dependent by NMR experiments, which has been explained in section 4.5 and Figure 4.5.1. The above result shows that FKBP25 interacts with plasmid DNA in a salt-dependent

manner which indicates that the binding force between FKBP25 and DNA is mainly electrostatic in nature.

Figure 4.3: Gel shift assay showing salt dependency for FKBP25-DNA binding. (A) Gel shift assay of single-stranded plasmid DNA alone (lane 1) and with FKBP25 (molar ratio 1:1 in lane 2 and 1:250 in lane 3) showing no binding to ssDNA. (B) Gel retardation assay of the FKBP25-plasmid complex in the presence of increasing concentrations of NaCl shows that the interaction of FKBP25 with plasmid DNA decreases with increasing concentration of NaCl. Lane 1 has DNA alone while lane 2-7 had DNA incubated with FKBP25 at increasing concentrations of NaCl (0, 100, 200, 400, 800, 1600 mM NaCl respectively). It can be observed that at 800 mM salt concentration, DNA is almost in free form and at 1600 mM NaCl (lane 7) the DNA is absolutely free from FKBP25 protein, indicating that FKBP25-DNA interaction was completely abolished.

4.6 Biophysical characterization of FKBP25-DNA interaction.

Since FKBP25 recognizes large dsDNA segments (plasmid DNA or linear DNA fragments), we tested FKBP25's ability to recognize oligonucleotides. For this purpose, we decided to use a 23-bp dsDNA oligonucleotide (referred to as DNA^{YY1}). Except for the three additional nucleotides at its 3' end, the sequence of DNA^{YY1} is same as that which can be recognized and bound by YY1, a well-known transcription factor (Wishart et al., 1994c). The purpose of choosing this sequence was to further explore the role of FKBP25 in enhancing the binding of YY1 with DNA^{YY1} and also the possibility of formation of a ternary complex of FKBP25, YY1, and DNA^{YY1} (Yang et al., 2001). We employed ITC, tryptophan quenching and NMR to characterize FKBP25-DNA^{YY1} binding.

4.6.1 ITC shows FKBP25 binds with oligonucleotide

Isothermal calorimetry (ITC) is a powerful technique to study the interaction of a protein with another protein, DNA or small molecule. The gradual mixing of titrant into titrate results in absorption or release of heat, which can be measured and used as an evidence for the binding of titrant and titrate. The data obtained from ITC contains a wealth of information as it provides information about binding affinity, the stoichiometry of binding and also changes in entropy and enthalpy.

Figure 4.4: Characterization of FKBP25-DNA binding by ITC. The raw data of heat changes (upper panels) and the processed curve fit (lower panel) are shown for 0.1 mM of 23 bp dsDNA^{YY1} titrated into 25 µM FKBP25 protein. ITC results of the FKBP25 titrated with DNA^{YY1} suggests that FKBP25 binds with DNA^{YY} and the binding affinity was estimated to be 1.23 ± 0.15 µM.

We employed ITC experiments to investigate the binding and binding energetics of FKBP25-DNA^{YY1} interaction. ITC experiments show that FKBP25 could bind to an oligonucleotide (DNA^{YY1}). The FKBP25-DNA^{YY1} interaction appears to be an endothermic process as suggested by an upward trend of the ITC titration peaks and the positive resultant integrated heat (Figure 4.4). The result also suggests a single binding site of FKBP25 on DNA^{YY1} with the complex formation being driven by positive changes in entropy. The estimated K_d value for FKBP25-DNA^{YY1} binding was $1.23 \pm 0.15 \mu\text{M}$, which indicates moderate affinity of FKBP25 towards DNA. The obtained values of ΔH and ΔS were $1.2\text{E}4 \pm 375.1 \text{ cal/mol}$ and 68.8 cal/mol/deg respectively.

4.6.2 Tryptophan quenching experiment shows that FKBP25 binds with oligonucleotide

Further to confirm the binding of FKBP25 with DNA^{YY1}, we also performed tryptophan quenching experiments. High concentration of DNA^{YY1} was gradually titrated into FKBP25 and the sample was excited at 290 nm and emission spectra from 300-420 nm were obtained. The result showed that the fluorescence intensity decreased with the increase in DNA^{YY1} concentration (Figure 4.5A). The observed change in fluorescence intensity might be due to a change in the environment of the indole ring of the tryptophan residue in FKBP25 upon DNA^{YY1} binding. The relative fluorescence intensity was estimated as $F_{\text{relative}} = (F_o - F) / F_o$ where F and F_o are the fluorescence intensities at 342 nm in absence and presence of DNA^{YY1} respectively. We also performed blank experiments in which buffer was titrated into FKBP25 using the same experimental set up as used for ITC of DNA^{YY1} FKBP25 binding. To avoid the effect of dilution, the relative fluorescence intensity obtained upon buffer dilution was subtracted from the corresponding fluorescence intensity obtained upon DNA^{YY1} binding. Finally, the calculated relative fluorescence intensity was plotted against the concentration of DNA^{YY1} to estimate the binding affinity (K_d) (Figure 4.5). The graph was plotted through ligand depletion method. The binding affinity of FKBP25 with DNA^{YY1} was estimated as $2.6 \pm 0.5 \mu\text{M}$ which is comparable with the K_d obtained by ITC (Figure 4.4).

Figure 4.5: Tryptophan quenching experiment showing FKBP25-DNA^{YY1} binding. (A) The fluorescence intensity of 5 μM FKBP25 decreases with increase in the concentration of DNA^{YY1} from 0 μM to 45 μM. The arrow indicates that the top most line represents free protein while the bottom one represents the highest amount of titrated DNA^{YY1} (45 μM). (B) The relative fluorescence intensity $[(F_0 - F) / F_0]$ of FKBP25 is shown as a function of DNA^{YY1} concentration where F and F_0 are the fluorescence intensities at 342 nm in absence and presence of DNA^{YY1} respectively. The plot was used to estimate binding constant of FKBP25-DNA^{YY1} interaction.

4.7 DNA^{YY1} binding site on FKBP25 revealed by NMR titration

The results obtained from gel retardation assay, ITC and tryptophan quenching experiment indicated that FKBP25 could interact with double-stranded DNA but not with single-stranded DNA. In order to confirm this binding and to probe the DNA binding interface of FKBP25, we performed NMR titration of FKBP25 with double-stranded DNA (dsDNA^{YY1}) or with single-stranded DNA (ssDNA^{YY1}). First of all, we collected the HSQC spectra of FKBP25 mixed with DNA^{YY1} in 1:1 molar ratio and found that many peaks were missing in the spectra. Hence, we obtained the TROSY-HSQC spectrum which was found to be better resolved than that of HSQC spectra. When we overlaid TROSY-HSQC spectra of free FKBP25 and FKBP25 mixed with dsDNA^{YY1}, we observed that some of the cross peaks were shifting and broadening (Figure 4.4.1). There was no evidence of multiple species. We could observe a slow exchange rate as residues were gradually shifting upon increasing concentration of DNA. Thus, consistent with our previous observation, NMR data also showed that FKBP25 can bind to DNA^{YY1}.

Figure 4.6: The NMR titration FKBP25 with double stranded DNA^{YY1}. The overlaid TROSY-HSQC spectra of FKBP25 without (red), with 1:1 (green) or 1:2 (blue) dsDNA^{YY1} show a gradual shift in some cross-peaks on DNA^{YY1} binding. CSPs of few residues are zoomed and shown in boxes at the left.

It was difficult to identify all the peaks which showed chemical shift changes upon binding in above mentioned overlaid spectra, as some of the peaks were overlapped with others. In order to correctly identify those residues which showed shift upon DNA^{YY1} binding, we performed one more titration of FKBP25 with DNA^{YY1} at 1:2 molar ratio. The overlay spectra of FKBP25 with DNA^{YY1} (1:1 and 1:2) or without DNA^{YY1}, was used to assign those residues which shows shift upon DNA^{YY1} binding (Figure 4.6). The chemical shift perturbations (CSPs) of all residues were calculated using the formula $\Delta\delta = [(\Delta^1\text{H})^2 + (\Delta^{15}\text{N}/5)^2]^{1/2}$, where $\Delta^1\text{H}$ and $\Delta^{15}\text{N}$ are changed in chemical shift of ^1H and ^{15}N respectively, upon DNA^{YY1} binding. Further, CSPs of all the residues were plotted and the plot demonstrated that the residues which showed significant CSP (> 0.05 , which is sum of average CSP and standard deviation) upon DNA^{YY1} binding belong mainly to the N-terminal domain (Figure 4.7). Surprisingly, we also observed some residues from C-terminal domain showing significant chemical shift perturbation upon DNA^{YY1} binding.

Taken together, we concluded that both N-terminal HLH domain and C-terminal FKBD could be involved in DNA recognition and binding. Those residues which showed significant changes in chemical shift were Glu18, Gln19, Lys22, Lys23, Asp24, Leu29, His32, Leu38, Ala39, Lys 42, Leu43, Ile47, Lys48, Ala54, Leu59, Asn64, His65, Leu66, Gln150, Lys156, Lys157, A159 and Lys160 (Figure 4.7) from the HLH domain. There were only six residues from FKBD (Gln150, Lys154, Lys155, Lys157, Ala159, and Lys160) which showed significant chemical shift perturbation upon DNA^{YY1} binding. Interestingly, all those six residues from the C-terminal FKBD are located in the 40s loop which suggested that FKBD also assists in DNA binding.

Figure 4.7: CSPs in the residues of FKBP25 upon DNA binding (a) Weighted CSPs for the ¹⁵N and ¹H resonance of FKBP25 after DNA binding; the lower black line represents the average CSP while the upper black line is the sum of average CSPs and standard deviation. Most of the residues which shifted upon DNA binding belong to HLH domain while some of them were from FKBD, indicating the possible role of HLH and FKBD in DNA binding.

The 40s loop of FKBP25 is unique as it is relatively long and bears a series of lysine residues (Figure 4.17) and hence we have renamed the ‘40s loop’ of FKBP25 as the ‘basic loop’. Apart from HLH and FKBD, the flexible loop connecting these domains also bears a few lysine residues but none of them showed significant CSP upon DNA^{YY1} binding indicating that this loop is not involved in DNA binding. In summary, FKBP25 binds with

DNA^{YY1} mainly through its HLH domain while the basic loop of FKBD could further assist in this binding and the long flexible inter-domain loop does not play any role in the FKBP25-DNA^{YY1} interaction.

Using the information obtained from the NMR titration, we mapped the FKBP25 DNA^{YY1} binding interface. The mapping of residues showing significant CSP's, onto the surface of FKBP25, revealed that there were two major binding surfaces to mediate this interaction. The first one was on the HLH domain and the other one was on the basic loop of FKBD (Figure 4.8).

Figure 4.8: DNA-binding surface on FKBP25 revealed by NMR. The DNA^{YY1}-binding surface of FKBP25 mapped by CSP results represented in surface (A) and cartoon (B) representations. The residues having chemical shift perturbation more than 0.07 are represented in red while those showing chemical shift perturbation between 0.05- 0.07 are shown in green.

4.8 FKBP25 binds with dsDNA^{YY1} in a salt-dependent manner and it does not bind to ssDNA^{YY1}

We already performed gel shift assay experiments to prove that FKBP25-DNA interaction is salt dependent and also that FKBP25 shows lower or no binding to single-stranded DNA, using plasmid DNA. In order to confirm the observation holds good for FKBP25-oligonucleotide as well, NMR titration of FKBP25 with DNA^{YY1} was performed at 1:1 molar ratio in buffer containing either 50 mM or 150 mM NaCl concentration. The overlay spectra of FKBP25 with DNA^{YY1} at 50 mM or 150 mM NaCl and without DNA^{YY1} showed that the CSPs were higher at 50 mM salt concentration with respect to 150 mM salt concentration. As the change in CSPs of residues of FKBP25 decreased with the increase of salt concentration, upon DNA^{YY1} binding, we concluded that FKBP25-DNA^{YY1} interaction is also salt dependent and hence mostly ionic in nature (Figure 4.9). This result is consistent with the result obtained from gel shift assay (Figure 4.3B).

Figure 4.9: NMR titration of FKBP25 with different salt concentrations showing the interaction of FKBP25 with DNA^{YY1} is salt dependent. The overlaid TROSY-HSQC spectra of free FKBP25 (red) and FKBP25 with DNA^{YY1} in 1:1 molar ratio either in 150 mM (green) or in 50 mM NaCl (blue). Spectra shows the chemical shift perturbation of residues are reduced when we decrease the NaCl concentration, which indicates that the FKBP25 and DNA^{YY1} interaction is mostly ionic in nature because of which binding is salt dependent.

In order to confirm that FKBP25 poorly binds with ssDNA, we performed an NMR titration experiment of FKBP25 in the absence and presence of ssDNA^{YY1}. The overlaid spectra of FKBP25 with dsDNA^{YY1} or ssDNA^{YY1} or without any DNA^{YY1} showed that there were almost negligible CSPs in all the residues of FKBP25 upon ssDNA^{YY1} binding. Consistent with gel shift assay data, our NMR experiments also showed that the FKBP25-ssDNA interaction is poor (Figure 4.10).

Figure 4.10: A comparative study of the binding of ssDNA^{YY1} and dsDNA^{YY1} with FKBP25. The overlaid TROSY-HSQC spectra of free FKBP25 (green), and FKBP25 titrated either with ssDNA^{YY1} (blue) or with dsDNA^{YY1} (red) in 1:1 molar ratio. The overlaid spectra demonstrate that cross peaks show significant shift upon dsDNA^{YY1} binding but show lesser or no shift upon ssDNA^{YY1} binding, indicating that FKBP25 has lesser or no affinity to ssDNA^{YY1}.

4.9: Mutational studies revealed critical amino acids of FKBP25 for the FKBP25-DNA interaction

When a protein binds with any other molecule (protein, DNA, RNA, drug etc), some of the cross peaks in the HSQC spectra shows a shift. Some of them could be directly involved in the interaction while others could be indirectly involved as they are localized in the close vicinity of the binding interface and show a chemical shift because of conformation change near the binding surface. It is difficult to identify residues that are directly involved in the binding based on CSPs data obtained by NMR. To identify those residues which are directly involved in binding, it is advisable to mutate suspected residues and then monitor the effect of the mutation. The binding of FKBP25 with DNA^{YY1} also showed CSPs of some residues as discussed earlier. To determine which residues are directly involved in FKBP25 DNA^{YY1} binding, six residues which showed significant CSPs

were site-specifically mutated as K22A, K23A, K42A, I47F, Q150E, and K157A. In this thesis, we used K22A, K42A, I47F, Q150E and K157A to represent the mutant forms of FKBP25 and FKBP25 to represent the wild-type protein. All mutant proteins were purified using the same protocol as that for wild-type FKBP25. Figure 4.11 confirms that these mutant forms could be purified similarly to the wild-type. All the mutant proteins were found to be soluble and could be purified with high yield. After obtaining the pure mutant proteins, we examined the DNA binding property of these mutants by gel shift assay using pSUMO plasmid, as was used before with wild-type FKBP25. Our result showed that the wild-type FKBP25 could bind DNA as observed by a significant retardation in mobility of DNA in agarose gel in the presence of FKBP25. The binding of wild-type FKBP25 causes a reduction in migration of DNA. On the contrary, if the mutated FKBP25's were used one would expect a normal DNA migration pattern, instead of a retarded migration. Though there were differences, none of the point mutations were able to completely abolish the protein-DNA interaction which shows that FKBP25 interacts with DNA at multiple points (4.12).

Figure 4.11: Purification of wild-type FKBP25 and its mutants. (A) The DNA sequence confirmation of the mutant plasmids. (B) Mutants were purified using the same protocol as for wild-type FKBP25 and run on 12 % SDS-PAGE gel. The result shows that the purified mutant of FKBP25 could be obtained with good purity and yield, similar to the wild-type.

With respect to wild-type FKBP25, K22A and K157A mutants showed better mobility of DNA, indicating reduced protein-DNA interaction. This indicates that the FKBP25 residues K22 and K157 can be directly involved in DNA binding. Although there was significant CSP in the cross peak of I47 in HSQC experiments (Figure 4.7), mutation of I47 to F47 did not have any effect on DNA binding, indicating that I47 does not make any direct contact with DNA. As I47F could not affect DNA binding, but still could show CSP upon DNA binding, it is possible that I47F does not make direct contact with DNA but it is present near the DNA binding surface; hence the change in conformation near the binding interface could result in CSP of I47F upon DNA binding. In conclusion, the mutation data confirmed that some of the residues of FKBP25 like K22, K23, K42, Q150, and K157 but not I47, could be directly involved in DNA binding.

Figure 4.12: Mutational studies of FKBP25 and its mutant. (A) Gel shift assay of wild-type and mutant FKBP25 showing that some of the mutants can reduce protein-DNA binding. Lane 1 had DNA (pSUMO) alone while other lanes had DNA incubated with wild-type or mutant FKBP25 (as indicated above each lane). All mutants except I47F shows reduced DNA binding. (B) Gel retardation of the plasmid DNA (pSUMO) with increasing concentrations of wild-type or K157A mutant FKBP25 at the DNA to protein molar ratios of 1:0, 1:1, 1:25, 1:125. The result suggests that residues K22, K23, K42, Q150 and K157 could be directly involved in DNA binding.

Further to confirm that the mutants have a less binding affinity to DNA with respect to wild-type FKBP25 and also to estimate the binding affinity of these mutants, we performed intrinsic tryptophan quenching experiments. FKBP25, K22A, Q150E, and K157A were used for this study. A total of 5 μM of these proteins were separately titrated with increasing concentrations on DNA^{YY1} until the saturation was achieved. The fluorescence intensity of FKBP25 or mutant gradually decreased as the concentration of DNA was increased; indicating wild-type FKBP25 and its mutant could bind to DNA. In order to do a comparative analysis of binding affinities of the mutants, we plotted relative fluorescence intensity versus DNA^{YY1} concentration (Figure 4.13A). The binding affinities (K_d) of mutants were estimated by fitting the curve using ligand fitted method. The Figure 4.13B indicates that the binding affinity of mutants was lesser than that of wild-type FKBP25. The estimated K_d of mutants (summarized in Table 4.1) shows that these mutants have almost 4-fold reduced DNA^{YY1} binding ability with respect to wild-type FKBP25. The reduced binding affinity of these mutants signifies the importance of residues like K22 and K157 in DNA binding. Taking the result of gel shift assay and tryptophan quenching experiment together, we concluded that residues like K22, K23, K42, Q150, and K157 make direct contact with DNA while the residue I47 is not involved in direct interaction with DNA.

Figure 4.13: Tryptophan fluorescence quenching experiments for the binding of FKBP25 and its mutants with DNA^{YY1} (A) The plot shows the representative binding curves for the interaction between FKBP25 or its mutants with DNA^{YY1}. The relative fluorescence intensities $[(F_0-F)/F_0]$ of 5 μM FKBP25, K22A, Q150E and K157A are shown as a function of DNA^{YY1} concentration. F and F_0 are the fluorescence intensities at 342 nm in the absence or presence of DNA respectively. (B) Using the plot of relative fluorescence intensity vs concentration, K_d of binding of FKBP25, K22A, Q150E and K157A with DNA^{YY1} were estimated and plotted. The result shows that binding affinity of DNA^{YY1} with mutants of FKBP25 is lower than that of wild-type FKBP25, which indicates that residues K22, Q150 and K157 could be directly involved in DNA binding.

Table 4.1: Summary of K_d of binding of wild-type FKBP25 and its mutants with DNA^{YY1}

Proteins	FKBP25	Mutant K22A	Mutant Q150E	Mutant K157A
K_d	$2.6 \pm 0.5 \mu\text{M}$	$13.4 \pm 1.6 \mu\text{M}$	$12 \pm 1.4 \mu\text{M}$	$12.3 \pm 1.4 \mu\text{M}$

4.10 Gel shift assay shows that both HLH and FKBD are required for DNA binding

In order to confirm the observation that both HLH and FKBD of FKBP25 are required for DNA binding, we performed gel shift assay. We purified full-length FKBP25, HLH domain and FKBD as described previously. These purified proteins were separately mixed with a pSUMO plasmid at 1:125 (DNA: protein) molar ratio and then was run on 1 % agarose gel. FKBP12 was used as negative control. The result of gel shift assay showed both of these individual domains had a very poor binding affinity to DNA with respect to full-length FKBP25 (Figure 4.14). This observation suggests that FKBP25 requires both its domain to bind to DNA efficiently.

Figure 4.14: Gel shift assay showing both HLH and FKBD are required for DNA binding. 300 ng pSUMO plasmid was mixed with FKBP25, HLH, FKBD or FKBP12 at 1:125 molar ratio and incubated for 30 min at room temperature. Lane 1 has DNA only, Lane 2-5 has full-length FKBP25, HLH, FKBD and FKBP12 respectively. The result shows that FKBP25 has a better binding than that of any of its individual domains suggesting FKBP25 requires both of its domains for binding. FKBP12 which was used as the negative control does not show any binding.

4.11 Intermolecular NOEs between FKBP25 and DNA^{YY1}

In order to understand the structural basis of nucleic acid recognition by FKBP25, we collected 3D-¹³C F1-filtered F3-edited NOESY spectra with ¹³C/¹⁵N labeled protein and unlabeled DNA^{YY1} and then attempted to observe NOE between FKBP25 and DNA^{YY1}. The assignment of DNA and NOEs were performed by Shin Joon from our lab. The result showed overlapping resonances and lack of sufficient unambiguous intermolecular NOE constraints. Despite the severely overlapped DNA resonances in the protein-DNA complex, a few unambiguous intermolecular NOEs were observed in 3D-¹³C F1-filtered F3-edited NOESY spectra. These unambiguous NOEs are Lys22 C^εH to H2'/H4' of T31 and H5 of C32 (Figure 4.15A and Table 4.2). The residues of DNA^{YY1} which exhibit intermolecular NOE with FKBP25 have been also shown in figure 4.15B and are labeled with asterisks on the sequence of the DNA^{YY1}. This result confirmed the direct involvement of residues like K22 and K157 of FKBP25 and some residues of DNA^{YY1} like T31 and C32. Later, we mapped the residues which showed NOE with FKBP25, on the DNA^{YY1} (Figure 4.15C)

and the mapping shows that these residues are present on one side of the DNA^{YY1} which could be the FKBP25 binding site.

Figure 4.15: Intermolecular NOE restraints between DNA^{YY1} and FKBP25. (A) Selective strips from ¹H-¹H planes of the 3D-¹³C F1-filtered, F3-edited NOESY-HSQC spectra recorded on a complex between unlabeled DNA^{YY1} and ¹³C-¹⁵N labeled FKBP25 in D₂O at 298K. Intermolecular NOEs between C^ε methylene groups of specific lysine residues (K22, K154, K155, and K156) located in the DNA binding regions and DNA proton resonances are shown as a rectangular box. (B) The sequence of dsDNA^{YY1} used for the assignment of intermolecular NOE restraints between FKBP25 and DNA^{YY1}. Residues which show the intermolecular NOEs with FKBP25 are shown by asterisks. The numbering of residues in dsDNA^{YY1} is given as 1-23 in sense strand and 24-46 on anti-sense strand both in 5' to 3' direction. (C) The residues of DNA^{YY1} which show intermolecular NOE with FKBP25 were mapped on DNA^{YY1}. Mapping shows that these residues are present on the same side of DNA^{YY1} to form FKBP25 binding site on DNA^{YY1}.

In order to estimate the changes occurred in DNA upon FKBP25 binding, we overlaid the spectra of DNA and DNA bound to FKBP25. We could not see significant difference in chemical shifts which suggests that there was a very little, if any, change in DNA upon FKBP25 binding. Because of lack of complete assignment of DNA, it was difficult to map all those residues of DNA which shifted upon FKBP25 binding.

4.12 Model of FKBP25-DNA complex

Using the information obtained from NOE, NMR titration, and mutagenesis data, we performed docking by HADDOCK. HADDOCK server is a very popular server to perform information-driven docking. In HADDOCK server, based on prior information, we can assign active residues which we believe to be involved in the direct interaction. We also need to assign passive residues, which are present near active residue and having the solvent accessibility of more than 50 percent. We can also choose for automatic passive residues selection, where the server automatically selects passive residues based on active residues. Based on the information we provide, HADDOCK performs a biased docking and provides the result as several docking models clustered based on energy parameters. To perform docking experiments, we used the solution structure of FKBP25 and DNA from the YY1-DNA complex (PDB ID – 1UBD) which has 20 bp. The reason for using this DNA was that it has the same sequence as the sequence of DNA^{YY1} except the fact that DNA^{YY1} had 3 extra nucleotides at 3' end. In addition, we could use this FKBP25-DNA^{YY1} complex model to study a possible ternary complex of FKBP25-DNA-YY1 which has been explained in detail in chapter 5. Because the DNA we used for docking is 20 bp while the DNA we used for all biophysical characterization, including NMR is of 23 bp, the numbering of the identical residues from these two DNAs are not same. Based on intermolecular NOEs, HSQC titration, and mutation data, we assigned K22, K23, K42, K48, Q150, K155, K156 and K157 as active residues for FKBP25. The selected active residues for DNA were A10, C23, T24, T25, C26, G32, A34 and G35 which correspond to residues A10, C29, T30, T31, C32, G38, A40 and A41 of DNA^{YY1} respectively. The result of docking was obtained as 10 clusters of protein-DNA complexes and inside each cluster, there were 4 models. Among all the models generated by HADDOCK, a model showing the least energy was selected as the final model for FKBP25-DNA complex and the energy

parameters have been summarized in Table 4.2. The FKBP25-DNA^{YY1} structure model revealed that both the HLH domain and the FKBD bind directly to DNA (Figure 4.16).

Figure 4.16: Structural model of the FKBP25-DNA complex (A) FKBP25-DNA complex structure model obtained from HADDOCK docking. The HLH domain, flexible loop, FKBD and basic loop are shown in red, blue, green and purple, respectively. The model shows that HLH domain binds to major groove and the basic loop from FKBD binds to the minor groove of DNA. (B) The FKBP25-DNA model with the residues of FKBP25 important for the interaction with DNA. DNA and FKBP25 are presented in a cartoon representation and residues are shown in a stick representation in yellow for HLH and cyan for a basic loop.

Consistent with the mutational study, the HLH domain binds with DNA through residues K22, K23, and K42 which form salt bridges with the phosphate backbone of DNA^{YY1} (Figure 4.16B). Likewise, the residues K154, K155, K156, K157 from the C-terminal FKBD, also forms a direct salt bridge with the phosphate backbone of DNA^{YY1} (Figure 4.16B). As observed from intermolecular NOE, residues of DNA^{YY1} like A10, G32, A34, and G35 make contact with charged residues of basic loop and residues like C23, T24, T25, C26, interact with HLH domain. Interestingly, in the model, we could observe that I47 is present in close proximity with DNA^{YY1} but not as close to make any contact which explains why we could see chemical shift perturbation of I47 upon DNA binding (Figure 4.2) while we could not see any change in binding affinity to DNA on gel shift (Figure 4.12) when we mutated from I47 to F27. Similar to I47 all other residues of FKBP25 which

showed significant CSPs on DNA binding were present in close proximity to the DNA, suggesting that the FKBP25-DNA structural model was consistent with the NMR titration data and mutagenesis data. Our model demonstrates a unique mechanism of binding of FKBP25 with DNA, as the N-terminal domain binds with the major groove of DNA while the basic loop of FKBP25 binds with the adjacent minor groove. We have shown earlier that the domain-domain interaction is poor, so it is also possible that in some cases the basic loop could bind to minor groove situated 3-4 minor grooves away from the major groove binding to the HLH domain. Although the binding of the N-terminal HLH to DNA was expected, the binding of FKBD with DNA through the basic loop was a unique feature. It is to be highlighted here that till date none of the FKBD of any FKBP's has been shown to bind with DNA. To further investigate why only FKBD of FKBP25 could assist in DNA binding, we performed both sequence and structural alignment of FKBD of FKBP25 with other FKBP's from human or other species. Sequence alignment shows that there is the insertion of a series of lysine residues (KKKKNAK) which is exclusively present in FKBD of only human FKBP25 (Figure 4.17A). Surprisingly these lysine residues are the same residues which we showed to be important for DNA binding. So these extra residues in the human FKBP25, only present in FKBD of human FKBP25, could show any contribution towards DNA binding. We also performed sequence alignment of human FKBD of FKBP25 with the FKBDs of homologs of FKBP25 from plasmodium (*PvFKBP25*), yeast (FRP3 and FRP4), plant (*AtFKBP53*) and fall armyworm (*PmFKBP46*) and the result suggest that these extra lysine residues are exclusively present in human FKBP25 and even the homologs of human FKBP25 from different species do not contain such a stretch of lysine residues (Figure 4.17B). These stretch of lysine residue is located in 40s loop of FKBP25 which makes this loop highly charged and extra-long. Further, we superimposed 40s loop of FKBDs of different human FKBP's or FKBDs of homologs of human FKBP25 from other species. The superimposed structures revealed that the basic loop of FKBP25 is relatively longer than that 40s loop of FKBDs from other human FKBP's. Even the structural alignment of FKBD of FKBP25 either from human or plasmodium or yeast homolog of FKBP25 shows that the long basic loop is unique to human FKBP25 (Figure 4.17C and D).

Table 4.2: Parameters used for HADDOCK docking and the statistics of final FKBP25-DNA^{YY1} model

Ambiguous interaction restraints	
Active residues (FKBP25)	K22, K23, K42, K48, Q150, K156 and K157
Passive residues (FKBP25)	Automatically selected by HADDOCK server
Active residues (DNA)	A10, C23, T24, T25, C26, G32, A34 and G35
Passive residues (FKBP25)	Automatically selected by HADDOCK server
Intermolecular NOEs between FKBP25 and DNA	K22-H ^ε / T31-H2', T31-H4', C32-H5 K154, K155, K156H ^ε / A10-H1', A40-H1', G41-H1'
Statistics of the final four best energy water-refined structures	
HADDOCK score	-142.5 ± 8.1
Energies	
Electrostatic	-692.6 ± 45.5 kcal/mol
van der Waals	-58.7 ± 2.3 kcal/mol
Ambiguous Interaction Restraint (AIR)	57.8 ± 27.7 kcal/mol
Buried surface area	1654.4 ± 26.2 kcal/mol
Backbone RMSD to the average structure on interface	0.8 ± 0.4
Ramachandran map regions ^a (in %)	79.6/17.0/1.5/1.9

^a Ramachandran map region is determined using the PROCHECK-NMR program (Lakowski et al, J. Biomol. NMR. 8, 477-486, 1996). Favored/ additionally allowed/ generously allowed/ disallowed regions are displayed.

Figure 4.17: Sequence alignment and structural comparisons of the basic loop of FKBP25 with different human FKBDs and homologs of FKBP25. (A) The sequence alignment of FKBD of human FKBP25 with FKBDs of other human FKBDs (FKBP12, FKBP2, FKBP5, FKBP8, and FKBP6) performed using T-Coffee. (B) The sequence alignment FKBD of the human FKBP25 with homologs of FKBP25 from plasmodium (*PvFKBP25*), yeast (FRP3 and FRP4), plant (*AtFKBP53*) and fall armyworm (*PmFKBP46*). The highly basic loop of FKBP25 is shown in the blue box. (C) Cartoon representation of the superposition of basic loop of FKBP25 (blue) with 40s loop of FKBP12 (magenta), FKBP2 (yellow), FKBP5 (slate), FKBP8 (orange) and FKBP6 (green). (D) Comparison of the basic loop of human FKBP25 (blue) with 40s loop of *PvFKBP25* (green) and FRP4 (red).

In conclusion, sequence alignment and structural comparison suggest that a stretch of lysine residues is exclusively present only in the basic loop of human FKBP25 which explains why FKBD of only human FKBP25 could show the property of the nucleic acid binding. Further, the structure of full-length FKBP25 revealed that this basic loop is situated away from the FK506 binding pocket and also present in close proximity with the N-terminal domain (Figure 3.19; chapter 3) so that it could assist the N-terminal HLH domain in DNA binding. Thus, our model for FKBP25-DNA complex suggests that the relatively long and unique basic loop of FKBP25 serves as a novel motif to aid nucleic acid-binding by FKBP25 and also that it is an adaptation of FKBP25 to have this basic loop for efficient DNA binding.

4.13 Paramagnetic relaxation enhancement (PRE) measurements

Paramagnetic relaxation enhancement (PRE) of ^1H is known to be used as a source for long-range distance information which cannot be derived from intermolecular NOE. PRE is being used as a powerful tool to determine the polarity of protein for DNA binding if we introduce a metal binding site in DNA. To obtain the PRE data for a protein-DNA binding, a new method was developed by Iwahara et al., in which they suggested introducing an EDTA labeled thymine in one of the strands of the DNA for metal binding.

To validate the model of the FKBP25-DNA complex generated by HADDOCK, we also performed PRE experiments using the above-mentioned method. We prepared two modified DNAs, which have the same sequences as DNA^{YY1}, each DNA having one EDTA labeled thymine. DNA-1 had EDTA labeling at position 5 while DNA-2 had EDTA labeling at position 27 (Figure 4.18A). In this way, we had two kinds of DNA, each labeled at distinct ends. The reason for preparing two different DNA was to identify and distinguish those residues of FKBP25 which are close to one end of DNA or the other end. Paramagnetic and diamagnetic states were obtained by generating DNA in complex with either Mn^{2+} or Ca^{2+} , respectively. ^{15}N TROSY-HSQC spectra then were acquired for FKBP25-DNA-1 and FKBP25-DNA-2 complexes in paramagnetic and diamagnetic states. To check whether spin labeling had an effect on FKBP25-DNA binding, the spectra from the complex with or without the spin label were superimposed. Because these superimposed spectra fit well, we concluded that spin labeling did not cause any changes

in FKBP25-DNA binding. Moreover, the overlaid spectra of the FKBP25-DNA-1 complex in paramagnetic and diamagnetic states showed that the intensities of some of the peaks decreased in the paramagnetic state (Figure 4.18B). The residues which showed reduced peak intensity or disappeared should be located close to EDTA labeled thymine of DNA-1. So further, we attempted to identify those residues which are close to the labeled end of DNA-1. We repeated the similar experiment with DNA-2 and also got a similar result.

Figure 4.18: PRE effect on DNA binding (A) The sequences of two modified DNAs with one strand having EDTA-labeled thymine either at position 5 (denoted as DNA-1) or at position 27 (denoted as DNA-2). Thymine with EDTA labeling is shown in red. (B) The overlaid ^1H ^{15}N TROSY-HSQC spectra of the FKBP25-DNA-1 complex obtained in paramagnetic (in red) and diamagnetic (in blue) states by chelating labeled EDTA with Mg^{2+} and Ca^{2+} , respectively. A section of the overlaid spectra has been zoomed and shown on the right side of spectra. The spectra show that in paramagnetic state, the peak intensity of several peaks decreased which indicates that these residues are present in close vicinity to the modified thymine of the DNA-1.

The ratios of the peak intensities for paramagnetic and diamagnetic states ($I_{\text{par}}/I_{\text{dia}}$) were estimated and plotted for all residues of FKBP25 (Figure 4.19A and B). PRE measurements for the FKBP25-DNA-1 complex showed that the $I_{\text{par}}/I_{\text{dia}}$ ratio was significantly attenuated for residues Glu18-Ile26 from the $\alpha 1/\alpha 2$ loop and $\alpha 2$ helix, Gly45-Asp57 from the $\alpha 3/\alpha 4$ loop, $\alpha 4/\alpha 5$ loop and $\alpha 4$ helix of HLH and also Gln150-Lys160 from $\beta 3/\beta 4$ loop (the basic loop) of FKBD (Figure 4.19A and B). In the paramagnetic state, the peaks of some residues, such as Lys22, Lys23, Lys48, Lys52, Thr53 and Ala159, completely disappeared, indicating that these residues are located closest to Mn^{2+} bound to DNA-1 (Figure 4.19A).

Figure 4.19: The plot depicts the ratio of peak intensities of all residues from paramagnetic to diamagnetic states for FKBP25 in complex with DNA-1 (A) or DNA-2 (B). The arrows in the plot show a stretch of residues that are important for DNA binding. (C) Residues of FKBP25 showing PRE effect were mapped on FKBP25-DNA model and in support of our FKBP25-DNA model; the map shows that those residues are present in close vicinity to DNA. The residues with an intensity ratio less than 0.4 are colored blue and red for the HLH domain and FKBD, respectively.

The PRE results supported the FKBP25-DNA model very well because all the residues that showed PRE effects were positioned in close proximity to DNA in the FKBP25-DNA complex model (Figure 4.19C). The Lys22, Lys23, Lys42 and Lys48 are those residues which showed maximum CSPs on DNA binding (Figure 4.7). These residues also showed ionic interactions with the DNA phosphate backbone, and in support of that model, the PRE experiments also showed maximum PRE effects for these residues. The peaks of FKBD residues Gln150-Lys160 were significantly attenuated, which confirmed that FKBD, through its basic loop, was also involved in binding with DNA, thus supporting the FKBP25-DNA model. Some residues from FKBD, but not those belonging to the basic loop, such as Lys118, Ile223, and Asp224, also showed significantly reduced peaks under paramagnetic conditions, which is logical because these residues are located close to the basic loop of FKBD. Surprisingly, the results of PRE experiments we obtained for DNA-1 and DNA-2 were found to be the same (Figure 4.19A). This observation could be explained by the fact that FKBP25 binds to DNA in a sequence-independent manner and any dT-EDTA-labeled end of DNA (either DNA-1 or DNA-2) could bind to any side of protein (either HLH domain or FKBD) and hence no difference was seen in the PRE results for binding of DNA-1 and DNA-2 to FKBP25. Thus, we could conclude that any end of DNA could bind with the HLH domain while the other end could bind with the FKBD of FKBP25. In conclusion, the result of PRE experiments validated our proposed model for FKBP25-DNA complex.

In a paper published by Yang et al. had shown that FKBP25 interacts with YY1 (Yang et al., 2001). In the EMSA experiment they performed to show FKBP25-YY1 binding, they could not observe any binding of FKBP25 with DNA. The possible reason could be the condition they used for EMSA would not show FKBP25-DNA binding. But in our study, by several biophysical methods, we have shown that FKBP25 interacts with DNA. This study has opened several new questions, like why FKBP25 interacts with DNA, is there any sequence specificity shown by FKBP25 to recognize DNA. These questions will be further explored in our lab.

Chapter 5

Role of FKBP25 in YY1-DNA binding; a modeling perspective

5.1 Aim and overview of study

Yin Yang 1 (YY1) is a well-known transcription factor which binds to DNA through its DNA-binding domain (DBD) consisting of four zinc finger domains (Figure 5.1A). The binding affinity of YY1 is poor with respect to other transcription factors and thus, it was suggested that YY1 may require some co-regulator proteins to enhance its binding affinity to DNA. FKBP25 was identified as a YY1 binding protein and was shown to enhance the DNA binding ability of YY1. The mechanism through which FKBP25 could help YY1 to enhance its binding to DNA remains elusive. In the previous chapter, we have shown that FKBP25 could bind DNA but the biological function of such binding is unclear. Here in this study, we tried to answer the question as to how FKBP25 could enhance the binding ability of YY1 to DNA. By answering this question, we also tried to present the biological significance of FKBP25 mediated DNA binding. Toward this end, we have mapped YY1 binding site on FKBP25 and generated a docking model of FKBP25-YY1-DNA. Finally, we have proposed how FKBP25 could function as a co-regulator of YY1.

5.2 Cloning, expression and purification of YY1-DBD

In order to characterize YY1-FKBP25 interaction, we cloned the DNA binding domain of YY1 (referred as YY1-DBD) into a pET29b expression vector (Figure 5.1A). We confirmed the positive clone by DNA sequencing followed by sequence alignment. After getting a correct clone, we optimized expression and purification of the recombinant YY1-DBD protein. In order to optimize expression, the clone was transformed into *E.coli* BL21 cells and induced with 0.5 mM IPTG for 1 h, 2 h and 4 h at 25 °C. After induction, the cells were lysed and loaded onto a 12 % SDS-PAGE gel. The gel picture shows that transformed cells were able to express the protein and the yield was highest after 4 h of induction (Figure 5.1B). After optimizing the expression of YY1-DBD, we tested its solubility in lysis buffer. Although some fraction of YY1-DBD was present in pellet fraction, we were able to get enough of protein in lysis buffer, indicating that the protein was well folded. Later we purified YY1-DBD using Ni-NTA column and procured almost 95 % pure proteins (Figure 5.1C).

Figure 5.1: The expression and purification of YY1-DBD. (A) The domain organization of YY1-DBD construct (residues 286 to 414 of YY1 protein). Each domain is colored and labeled with their corresponding residue numbers. (B) The optimization of expression of the YY1-DBD protein. Cells were induced with 0.5 mM IPTG for 1, 2 and 4 hours and the lysed cells were run on 12 % SDS-PAGE gel. The gel picture shows that cells were able to express YY1-DBD (indicated by red arrow) and the expression level was highest after 4h of induction. (B) The purification of the YY1-DBD protein. Gel picture shows that YY1-DBD was expressed and it was present in the supernatant fraction, indicating that protein was soluble. Pure protein was collected in the eluted fraction.

Later we performed 1D NMR experiment to check the folding of purified YY1-DBD. The 1D NMR spectra showed well-dispersed peaks confirming that the purified YY1-DBD was well folded in solution (Figure 5.2).

Figure 5.2: 1D NMR spectra of recombinant YY1-DBD. Peaks are well dispersed revealing that the protein is in a folded state.

5.3 The YY1-binding surface on FKBP25 revealed by NMR titration

It was shown that FKBP25 interacts with YY1 and residues 300-333 of YY1 were found to be important for such an interaction (Yang et al., 2001). In order to map the YY1 binding site on FKBP25, NMR ^{15}N TROSY-HSQC experiments were performed either with YY1-DBD protein or with YY1 peptide (a peptide containing residues 300-333 of YY1). In both cases, NMR samples were supplemented with 0.1 mM ZnCl_2 . The result showed that both YY1-DBD and YY1 peptide were able to bind to FKBP25 in a similar way as CSP's were observed for the same set of residues in FKBP25 in either case. This indicated that YY1 peptide is sufficient to bind FKBP25, thus we decided to use YY1 peptide for other NMR titration experiments. We performed NMR titration of FKBP25 with YY1 peptide at 1 : 1, 1 : 2, 1 : 5, and 1 : 10 molar ratios (Figure 5.3). The overlaid HSQC spectra showed a gradual shift in peaks, with a concomitant increase in YY1 concentration.

Figure 5.3: NMR titration of FKBP25 with 300-333 YY1 peptides. (a) The overlaid HSQC spectra of FKBP25 without (red) or with YY1 peptide at molar ratios of 1:1 (yellow), 1:2 (purple), 1:5 (green) or 1:10 (blue). Some of the cross peaks show gradual shifting upon peptide binding. Chemical shift perturbation of some residues from the N-terminal domain and C-terminal domain are zoomed and shown in upper and lower boxes respectively.

It is evident from the spectra that most of the residues which shifted upon YY1 peptide binding belong to HLH domain which is consistent with previous reports (Helander et al., 2014). The residues from HLH domain, which show significant chemical shift perturbation upon YY1 peptide binding are Gln30-His32 (belonging to α_2), Leu38-Ala39 (belonging to α_3), Asn64-Leu66 (belonging to α_5) and Gly74 (Figure 5.4).

Figure 5.4: Chemical shift perturbation on YY1 binding. Bar diagram of weighted chemical shift perturbations versus residue number of FKBP25 upon YY1 peptide binding. The black line represents average CSP. Residues with significant chemical shift perturbation have been labeled.

Interestingly, we also observed that some of the residues from the FKBD (His132, Cys133, Leu162, and Asp222) also showed CSPs upon YY1 peptide binding (Figure 5.5A). These residues were present as a patch located on the rear side of the FK506-binding pocket, facing the HLH domain (Figure 5.5A, middle and right panels). This indicates that FKBD may also be involved in YY1 binding. It has been shown that the presence of FK506 does not affect the binding affinity of FKBP25 with YY1 (Yang et al., 2001). This is possible because the structure of full-length FKBP25 showed that the FK506 binding pocket faces opposite to the HLH domain. So YY1 can bind with both HLH domain and a small patch on FKBD in such a way that it does not interfere with FK506 binding.

Figure 5.5: Mapping of the YY1-binding site on FKBP25. The mapped YY1-binding site in FKBP25 is presented in cartoon (A) and surface (B) representations. The model (in the middle panel) has been rotated 90 ° either on the horizontal axis (left panel) or on the vertical axis (right panel) to show the complete binding site. The YY1-binding site residues with CSP > 0.17 and those between 0.1 -0.17 are colored in red and green respectively. The mapping of the YY1 binding site shows that YY1 mainly interacts with $\alpha2$ and $\alpha5$ of the HLH domain of FKBP25.

5.4 Comparison of DNA and YY1 binding sites on FKBP25

We already showed that FKBP25 binds with DNA as well as with YY1. The binding of YY1 affects helices $\alpha 2$, $\alpha 3$ and mainly $\alpha 5$ of FKBP25 while the DNA binding mainly affects $\alpha 2$ and $\alpha 4$, which indicates that there could be some overlap in the DNA and YY1 binding sites on FKBP25. In order to investigate the possibility of a ternary complex formation by FKBP25, YY1 and DNA, we thoroughly compared the YY1 and DNA binding sites on FKBP25. In figure 5.6, we mapped the YY1 binding site on FKBP25-DNA model, and the map shows that the YY1 binding site is present close to DNA binding site with some possible overlaps. It suggests that YY1 may interfere with DNA binding or vice versa.

Figure 5.6: The YY1-binding sites on the FKBP25-DNA complex model. A portion of the YY1-binding site overlaps with the DNA-binding site (middle panel) but the major section of the YY1-binding site does not overlap with the DNA-binding site (left panel and right panel).

5.5 NMR competition experiment

As we observed that the binding surface of FKBP25 for DNA or YY1 has some overlaps (but not complete overlap), we next questioned whether YY1 can compete with DNA for FKBP25 binding or not. To this end, we performed several NMR titration experiments of ^{15}N FKBP25 with DNA. The idea was to obtain HSQC spectra upon 1:1

DNA binding and then to add YY1 in increasing molar ratios from 1:1 to 1:10, to observe the pattern of chemical shifts of the residues specific to DNA or YY1 binding. The HSQC spectra for free FKBP25, FKBP25-DNA (1:1), FKBP25-DNA-YY1 (1:1:1, 1:1:2, 1:1:5 and 1:1:10 molar ratio) were obtained. As expected, FKBP25-DNA complex showed a shift in residues specific to DNA binding. Later, when we added YY1 to the FKBP25-DNA complex (1:1 molar ratio), we could observe that the residues which are specific to DNA binding did not show any change in chemical shifts due to the addition of YY1 at 1:1:1 molar ratio. Then we gradually increased YY1 concentration to 1:1:10 molar ratio. When we overlaid all the spectra, we realized that residues important for DNA binding gradually shifted back to the position where they were before DNA binding (which means the free FKBP25 state) (Figure 5.7). In the overlaid spectra of free FKBP25 and FKBP25-DNA-YY1 (1:1:10) complex, there was no shift in those residues of FKBP25, which showed shift upon DNA binding. This observation suggests that when YY1 and DNA are present in same concentration (1:1:1 molar ratio), YY1 cannot affect FKBP25-DNA binding, but if we increase the concentration of YY1 to 10 fold (1:1:10), YY1 is able to completely replace all the DNA from FKBP25, as at this concentration we could not observe any CSP's of the residues of FKBP25 specific to DNA binding. Also at 1:1:10 molar ratio of FKBP25-DNA to YY1, the residue specific to YY1 could show shift, indicating that at 1:1:10 molar ratio, FKBP25 and YY1 forms a binary complex. Thus, we concluded that at high concentration, YY1 can compete with DNA for FKBP25 binding and forms a binary complex with FKBP25. This observation could be explained as FKBP25 has a stronger binding affinity to DNA than YY1 at 1:1 molar ratio. This is the reason why YY1 cannot replace DNA from FKBP25 at 1:1 molar ratio, but when we increase the concentration of YY1 to 1:1:10, YY1 can completely abolish DNA-FKBP25 interaction. This observation is also consistent with NMR data which shows that FKBP25 may have some overlap for DNA and YY1 binding (Figure 5.6).

Figure 5.7: NMR study showing competition between DNA and YY1 for FKBP25 binding. Overlaid ^{15}N TROSY-HSQC spectra of FKBP25 (in black), FKBP25-DNA^{YY1} complex (1:1 molar ratio; in blue) and FKBP25-DNA^{YY1}-YY1 peptide complex (1:1:10; in red). Zoomed sections (on the left) depict some residues which show chemical shift perturbations upon DNA binding (indicated by black arrows). It could also be observed upon addition of the YY1 peptide (red peaks) at 10 molar excess, the same peaks (blue) shift back to the free protein (black) state, (indicated by green arrows). Zoomed section within the spectra shows that the cross peaks of FKBP25 specific to YY1 peptide binding show enhanced CSP's upon addition of 10-molar excess concentration of YY1 peptide to the FKBP25-DNA^{YY1} complex. This indicates that at 10-molar excess concentration, the YY1 peptide can abolish the interaction of FKBP25 with DNA^{YY1} and simultaneously form a binary complex (FKBP25-YY1 peptide) with FKBP25.

5.6 ITC experiments for the binding of YY1-DBD either with DNA or FKBP25

To further understand the interaction dynamics of FKBP25, YY1-DBD and DNA^{YY1} it was important to estimate the binding constant for the one to one interaction of all three molecules (FKBP25, DNA^{YY1}, and YY1-DBD). To this end, several ITC experiments were performed. For the interaction of YY1-DBD with DNA^{YY1}, we titrated 0.2 mM DNA^{YY1} into 50 μ M of YY1-DBD. The result of ITC experiment showed that the binding was exothermic and enthalpy driven as the heat change was negative. The estimated K_d was $0.39 \pm 0.6 \mu$ M which was obtained by one binding site fitting model (Figure 5.8). The Summary of the binding affinity and thermodynamic parameter associated with binding of FKBP25 and YY1 with DNA^{YY1} are summarized in Table 5.1. Later we performed ITC experiments for the binding of FKBP25 with YY1-DBD. We observed that the heat change upon binding was very less and the K_d was not measurable, indicating a very weak binding of FKBP25 and YY1-DBD. Consistent with the observation made by NMR competition experiment, the comparison of binding affinities suggests that FKBP25 has a stronger affinity for DNA^{YY1} with respect to YY1-DBD while the binding affinity of DNA^{YY1} and YY1-DBD is strongest among the three.

Table 5.1: Thermodynamic parameter of interaction of FKBP25 and YY1 with DNA^{YY1}

Interacting partners	K_d (μ M)	ΔH (cal/mol)	ΔS (cal/mol/deg)
FKBP25 and DNA^{YY1}	1.23 ± 0.15	$1.246E4 \pm 375.1$	68.8
YY1-DBD and DNA^{YY1}	0.39 ± 0.6	-7551 ± 652	3.96

Figure 5.8: ITC measurements of binding of YY1-DBD to DNA^{YY1} or FKBP25. Upper panel represents the raw data for the binding of YY1-DBD to DNA^{YY1}. The lower panel shows integrated peak as a function of DNA^{YY1} to YY1-DBD molar ratio. In order to obtain the binding constant, data was fitted to the one-site binding model. The binding affinity has been indicated for the binding of YY1-DBD to DNA^{YY1}.

5.7 The ternary complex of FKBP25-YY1-DNA

The binding affinity of YY1 to DNA was shown to be in a micromolar range which is relatively low in comparison to other transcription factors bearing zinc-finger domains, which usually falls in the nanomolar range. As the binding affinity of YY1 is relatively low, it was suggested that other co-regulator proteins may be needed to assist and improve YY1 ability to bind DNA. For example, INO80, an ATP-dependent chromatin-remodeling complex could enhance the transcriptional activation mediated by YY1. Similarly, it was shown that FKBP25 was required for enhancing the transcriptional repression activity of YY1. The increased repression activity was a result of the increased binding affinity of YY1 to DNA by an unknown mechanism. Here, we propose that FKBP25 serves as a co-regulator and it forms a ternary complex with YY1 and DNA, where all three molecules

interact with each other. Such ternary complex formation could stabilize YY1 on DNA and thus could improve the affinity of YY1 to DNA. This hypothesis was based on some observations as follow. (1) It was shown that YY1, through its first zinc-finger (residues 300 to 333), interacts with HLH domain of FKBP25. Based on the co-crystal structure of YY1-DNA complex it was suggested that the second and third zinc fingers of YY1 are important for DNA sequence recognition and binding. The first zinc-finger is relatively loosely bound to DNA because the first zinc-finger of YY1 makes a single base contact to DNA while other zinc-fingers were shown to make multiple contacts. This explains why only the first zinc-finger could bind with FKBP25, not others. (2) In the YY1-DNA complex structure, there is a scope for the protein to bind both DNA and YY1 zinc-finger 1 (Figure 5.9A). (3) Our NMR data suggest that FKBP25 has an exclusive binding site for DNA or YY1 and these two binding sites are located in close vicinity on FKBP25. In addition, it could be observed that the interacting regions of DNA are almost mutually exclusive for FKBP25 and YY1 (Figure 5.12B). Based on these observations, one can assume that these three molecules could form a ternary complex.

To understand how FKBP25 could make a ternary complex with YY1 and DNA, we performed a HADDOCK docking. For docking, we used the YY1-DNA crystal structure (PDB ID- 1UBD) and NMR solution structure of FKBP25. HADDOCK generated 40 models which were clustered into 10 clusters. We picked the final model from cluster 2 which had the maximum HADDOCK score. The model revealed that in the ternary complex all three molecules also interact with each other by making one to one contact. The zinc fingers 1 to 4 of YY1 wraps around DNA through major grooves and FKBP25 occupy the exposed DNA in between the zinc finger 1 and 4 by interacting to both DNA and zinc-finger 1 (Figure 5.9B, C, and 5.10A).

Figure 5.9: A model for the FKBP25-DNA^{YY1}-YY1-DBD ternary complex. (A) The crystal structure of YY1 in complex with DNA. It could be observed that in between two ends of YY1, DNA is available to interact with any other protein. (B) The final model of FKBP25-DNA-YY1-DBD ternary complex generated by HADDOCK. The model suggests that FKBP25 finds a place in between the ends of YY1 and fits into the exposed DNA of the YY1-DNA complex. The complex structure was represented in the surface model and DNA, YY1, and FKBP25 was colored with brown (looks like grey), purple and green respectively. The model was shown in two different orientations (rotated 90 ° along the vertical axis). (C) Another representation of the docking model where YY1-DNA complex is shown as surface and FKBP25 as cartoon mode, revealing how FKBP25 fits into the exposed DNA.

Figure 5.10: The model of ternary complex and the details of interactions. (A) The ternary complex model is shown in cartoon mode in which DNA, YY1, and FKBP25 are shown in brown, purple and green colors respectively. The model shows that zinc finger 1 of YY1 (which is not tightly bound to DNA) interacts with helix $\alpha 5$ of HLH of FKBP25. On the other hand, $\alpha 3$ and $\alpha 4$ of HLH of FKBP25 interact with DNA and thus forms a ternary complex. A closer look at the interactions between $\alpha 4$ of FKBP25 and DNA (B) and interactions between $\alpha 5$ of FKBP25 and zinc finger 1 of YY1 are shown by representing interacting residues in stick mode. The model shows that residues H65 of FKBP25, which showed maximum CSP upon YY1 binding in NMR titration, interact with M306 of YY1. Similarly, K42 and K52 of FKBP25 interact with DNA.

The zinc-finger 1 binds with HLH of FKBP25 mainly through the helix $\alpha 5$ (Figure 5.10A). H65, the residue of HLH which showed maximum CSP upon YY1 binding (Figure 5.4), interacts with M306 of zinc-finger 1 of YY1, which explains why H65 showed maximum chemical shift (Figure 5.10 C). The $\alpha 4$ of FKBP25 which was shown to be important for DNA binding (Figure 4.4.3) retained its interaction with DNA in the ternary complex model (Figure 5.10A) as well. Residues K42 and K52 of FKBP25 which shift significantly upon DNA binding revealed their probable interactions with DNA (Figure 5.10 B) in the HADDOCK model. In this way, DNA binds with all zinc fingers of YY1 and also with $\alpha 4$ of FKBP25 while zinc-finger 1 of YY1 interacts with $\alpha 5$ of FKBP25 to form a ternary complex. Such ternary complex formation could stabilize YY1 on DNA and thus could improve the binding affinity of YY1 on DNA.

Earlier similar ternary complex formation has been shown, where Pax5, a paired box family protein, interacts with Ets-1, a transcription regulator, and both these proteins bind to the same DNA to form a ternary complex (Austin et al., 1994)(Figure 5.11).

Figure 5.11: The structure of the ternary complex of Pax5-Ets1-DNA. The ternary complex structure shows that Ets-1 (green color) interacts both with DNA (orange) and Pax5 (pink) whereas Pax5 also interacts with both Ets-1 and DNA to form a stable ternary complex (PDB ID – 1K78).

5.8 FKBP25 may act as recruitment factor for YY1

In support of the previous study showing YY1 can bind FKBP25 even in the absence of DNA (Yang et al., 2001), our ITC result also shows that FKBP25 can make a binary complex with YY1 with a lower binding affinity. Here, we propose a model to explain how FKBP25 could help YY1 to get recruited onto DNA for its transcriptional repression activity. FKBP25 could bind to YY1 first (Figure 5.11A) and then this binary complex searches for the DNA binding sequence for YY1 (as both YY1 and FKBP25 have a comparable affinity to DNA of 1.2 μ M and 0.39 μ M respectively). After reaching to transcription repression site, this binary complex could form a ternary complex in the above-mentioned fashion (Figure 5.8 and 5.9). Finally, after transcriptional repression activity, FKBP25 can release itself from the DNA but somehow can still retain binding with YY1 alone as our NMR titration (Figure 5.7) suggests that an increase in YY1 concentration abolishes FKBP25-DNA interaction (Figure 5.11C). Apart from this, it is also possible that these two proteins can independently interact with DNA and then form a ternary complex. However, given the limitations of these predicted models more studies are required to further understand the molecular mechanism governing the interactome among these macromolecules forming the ternary complex.

Figure 5.12: A speculative mode of action of FKBP25 if it acts as a helper protein in enhancing YY1 affinity with DNA. (A) FKBP25 (HLH domain in red and FKBD in green) binds to residues 300-333 (blue) of YY1 (cyan). The interacting residues, mapped using NMR titration, in FKBP25 are indicated in yellow. (B) FKBP25 then may electrostatically recognize DNA (the interaction region in DNA is colored in gray), thereby enabling YY1 to bind the DNA with higher affinity. In this model, it could be observed that the interacting regions of FKBP25 and YY1 with DNA are almost dissimilar; implying that these three could also co-exist as a ternary complex or be part of a multi-subunit complex or (C) the FKBP25 may release itself from DNA leaving YY1 binary complex with FKBP25.

In conclusion, we have showed how FKBP25 could form a ternary complex with YY1 and DNA and this ternary complex formation could assist YY1 for its transcriptional activity. In a similar way, FKBP25 could also help in transcriptional regulation by assisting other transcription factors.

Chapter 6

Conclusion

6 Conclusion

In this study, we solved the crystal structure of FKBD25 in complex with FK506 drug at 1.8 Å resolution. The structure of FKBD25 in FKBD25-FK506 complex has similar folds as in the previously reported FKBD25-rapamycin complex. By doing structural comparison of FKBD25-FK506 complex with either FKBD25-rapamycin complex or with FKBP12-FK506 complex, we tried to understand the reason for higher affinity of FKBD25 to rapamycin with respect to FK506. The pyranose ring of FKBD25 shows hydrophobic interaction with residues like A206, I208, F145, Y198, and D146. Like other FKBP, FKBP25 also forms four hydrogen bonds with FK506. In comparison to FKBP12, some of the residues like Arg42, Phe46, Glu54, His87 and Ile90 were replaced in FKBP25 by Asn158, Leu162, Lys170, Gln203 and Ala206 respectively. In the case of a substitution of His87 from Gln203, the interaction between Gln203 and pyranose ring of both FK506 and rapamycin were abolished which could reduce binding affinity of FKBP25 to both of FK506 and rapamycin. Another striking difference in FKBP12 and FKBP25 is the substitution Glu54 to Lys170 which results in two extra hydrogen bonds between the side chain of Lys170 of FKBP25 and rapamycin. This substitution could be the main reason for higher binding affinity of rapamycin to FKBP25. Thus our FKBP25-FK506 complex structure featured the importance of Lys 170 for the selectivity of rapamycin over FK506.

In order to solve the structure of full length FKBP25, we attempted to crystallize FKBP25 but unfortunately we were unsuccessful in generating a good diffracting crystal, so we next resorted to solve the structure of FKBP25 by NMR methods. A snapshot of the 1D and 2D HSQC spectra was found to be well dispersed which encouraged us to pursue the determination of the solution structure of FKBP25. To this end, we almost fully assigned the back bone and side chain amino acid composition of FKBP25 using several NMR experiments. Except for 7 non-proline residues (M1, A2, K56, K110, T151, A153 and K200) whose amide peaks were missing, the ^1H and ^{15}N chemical shifts of all residues of FKBP25 were assigned. Almost 98.6 % completeness was achieved by assigning ^{13}C chemical shifts for 213 non-proline residues. 212 out of 213 C_α , 198 out of 199 C_β and 206 out of 213 CO were assigned. We further assigned the side chain of all residues of FKBP25. Almost 97.6 % of all non-labile aliphatic ^1H and ^{13}C resonances and 95.2 % of ^1H resonances of all aromatic side chains were assigned. Finally, we calculated the structure

of FKBP25 and the solution structure showed that the structure of HLH and FKBD was almost same as previously determined individual structures of HLH and FKBD respectively. Interestingly, full length FKBP25 showed an interaction between its HLH and FKBD. There were hydrogen bond formations between the backbone amides of Val5 and Gln7 and the side chain oxygen atoms of the Thr138 and the Glu217, respectively. In addition, we could also observe an ionic interaction between Arg8 and Glu219. Such inter-domain interaction was also confirmed by titrating FKBP25 with rapamycin which caused CSPs (chemical shift perturbations) in the residues of both FKBD and HLH domain. The deletion of FKBD from the full length FKBP25 could show changes in chemical shift of some residues of HLH domain, but we could not observe significant CSP in residues like Q7 which showed NOE with FKBD. The relaxation data suggested that the domain-domain interaction is a weak interaction and both domains have some sort of flexibility. Such weak domain-domain interaction could impact the function of FKBP25 as a DNA binding protein or other protein interacting protein. The structure of full length FKBP25 also showed that the loop which connects both HLH and FKBD domains is very flexible and dynamic in nature. It has been speculated that this loop may also help in interaction with other proteins but there are no substantial reports yet supporting this theory.

FKBP25 is the only FKBP25 in the FKBP protein family which shows interaction with nucleic acid. Nucleic acid reorganization is an interesting feature of FKBP25, as FKBP25 is known to interact with several chromatin-associated proteins. Although it was shown that FKBP25 could be trapped on DNA matrix, very less is known about the FKBP25-DNA interaction. To this end, we did a detailed study of FKBP25 mediated DNA binding. First of all we mapped all charged residues on FKBP25 and we found that these charged residues make patches on the same side of both HLH and FKBD, which could serve as a DNA binding surface. As a proof of concept, we performed gel shift assay using plasmid DNA as a possible binding target of FKBP25. The gel shift assay showed that FKBP25 could bind plasmid DNA and this binding resulted in slow migration of the FKBP25-DNA complex with respect to free DNA. We performed similar experiments with other plasmids and also with the DNA ladder and obtained similar results which suggested that FKBP25 could bind DNA, albeit in a sequence independent manner. We later ascertained that this interaction was salt dependent which indicates that FKBP25-DNA

interaction is mostly electrostatic in nature and therefore sequence independent. After confirming that FKBP25 could bind large oligonucleotides like plasmid DNA, we tested whether FKBP25 could also bind to short oligos. For that purpose, we used a 23 bp DNA as a binding target and performed ITC and tryptophan quenching experiments on this protein-oligo complex. Both these experiments demonstrated that FKBP25 could interact with short oligos and the binding affinity was estimated to be 1.2 μ M and 2.4 μ M respectively. As DNA binding proteins can recognize either dsDNA or ssDNA or both, we next questioned which one was the preferred target of FKBP25. Both gel shift assay and NMR titration showed that FKBP25 could not bind ssDNA which suggests that FKBP25 can only recognize the secondary structure of dsDNA. Also, as there is no available information about how and where DNA could bind FKBP25, we attempted to map DNA binding site on FKBP25. NMR titration studies revealed that the DNA binding site on FKBP25 includes residues from both HLH and FKBD, facing the same side of FKBP25. Based on NMR titration studies, we mutated some residues of FKBP25 and the single amino acid mutants showed almost four-fold reduced binding than wild-type protein indicating the direct involvement of these residues in protein-DNA interaction. Later we also observed some unambiguous NOEs between FKBP25 and DNA. In order to understand the mode of binding of DNA to FKBP25, we performed HADDOCK docking. The best-fit model of FKBP25-DNA complex showed that both HLH and FKBD are involved in DNA binding through the major groove and minor groove of DNA respectively. The charged residues of N-terminal HLH make a salt bridge with the phosphate backbone of DNA. We observed that the 40s loop of C-terminal FKBD25 was also involved in DNA binding. This is an intriguing feature of FKBP25 as all other FKBP25s also bear the 40s loop and FKBD but none of them have been shown to bind DNA. Further investigation showed that the 40s loop of FKBD25 is relatively long and the extra residues in this loop are mostly lysine (KKKKNAK) thus we renamed it as the 'basic loop'. The sequence alignment analysis of human FKBP25 with FKBP25 homologues in other species, as well as with the other human FKBP25s suggested that this feature is exclusively present in human FKBP25. Our previous data from the structure of full length FKBP25 demonstrates that the basic loop of FKBD is present in close proximity of the N-terminal HLH domain which further substantiates our other observation that both the basic loop and the HLH domain cooperate

with each other to recognize DNA. Gel shift assay showed that individually, these domains of FKBP25 had very less binding to DNA with respect to full length FKBP25, suggesting that the cooperativity of these two domains enhances and facilitates DNA binding. Although this study has served to shed some light on the mechanism of DNA binding to FKBP25, it has spawned further questions and hypotheses regarding the biological significance of why FKBP25 interacts with DNA. There are several possible consequences of FKBP25-DNA binding. It was suggested that FKBP25 may have role in DNA repair, as it binds with proteins such as PARP1 and RPA which participate in DNA repair. Our report that FKBP25 interacts with DNA in a sequence-independent manner supports this hypothesis that FKBP25, as part of a protein complex, could be involved in DNA repair, although at this stage, this is purely hypothetical and open to further exploration. Another possible role for FKBP25-DNA interaction is in chromatin remodelling as it can also interact with chromatin associated proteins and also with DNA.

In order to further explore the biological relevance of FKBP25-DNA binding, we looked into the FKBP25-YY1 interaction. YY1 is a zinc finger transcription factor and the gene repressional activity of YY1 was shown to be increased in the presence of FKBP25. Here we proposed that FKBP25 can make a ternary complex with YY1 and DNA in such a way that all three moieties can make direct contact with each other and thus stabilize YY1 on to DNA to enhance the gene repression activity of YY1. To this end, we characterized the FKBP25-YY1 binding first. The NMR titration analysis of FKBP25 with YY1 revealed the YY1 binding site on FKBP25. The YY1 binding site was almost exclusive to DNA binding site on FKBP25, which suggested a possibility of ternary complex between FKBP25-DNA and YY1. Later we performed HADDOCK docking between FKBP25 and YY1-DNA complex. The docking model showed that YY1 binds with DNA through its zinc finger 2/3, and it binds with FKBP25 through its zinc finger 1. N-terminal HLH domain of FKBP25 binds both with DNA and YY1 through its distinct DNA and YY1 binding site respectively. In this way, FKBP25 could stabilize YY1 on DNA and thus improve the DNA binding ability of YY1 and transcriptional repression activity of YY1.

Later to understand how FKBP25 forms a ternary complex with YY1 and DNA, we determined the binding affinity of YY1 with FKBP25 and DNA. The binding affinity

of DNA with YY1 was 3 fold higher than FKBP25 binding while the FKBP25-YY1 binding affinity was found to be very poor. Later we performed DNA competition experiment by NMR titration of FKBP25-DNA complex with YY1. The result of this experiment shows that YY1 can compete with DNA and at higher concentration (almost 10 fold higher) of YY1, FKBP25-DNA interaction gets completely abolished and YY1 forms a binary complex with FKBP25. Based on these observations we proposed a model to explain the dynamics of interaction of FKBP25, YY1 and DNA. FKBP25 and YY1 can make a binary complex in the cytoplasm or nucleus and then FKBP25 can bind DNA in a sequence-independent manner and scan for the YY1 binding site on DNA. At the YY1 binding site on DNA, the ternary complex formation is accomplished which could stabilize YY1 on DNA. After completion of transcription repression, the interaction of FKBP25 could be abolished which leaves FKBP25 as part of the binary complex with YY1 and thus reduces transcriptional repression activity of YY1. In conclusion, we proposed FKBP25 as a co-regulator of YY1 and suggested that the ternary complex formation could improve the binding affinity of YY1 to DNA for transcription repression.

In summary, we have solved the crystal structure of FKBP25 in complex with the drug FK506, solved the NMR structure of full length FKBP25, characterized the DNA binding property of FKBP25 in detail and finally tried to explain the impact of FKBP25-DNA interaction on YY1 mediated gene repression.

Our results have opened up several new avenues of exploration, mainly to answer several questions which will be addressed in our lab in the future. Some of them are as follows: (1) Can FKBP25 make a ternary complex with YY1 *in vivo* and how this ternary complex formation could help in YY1 mediated gene repression? (2) Whether FKBP25-DNA interaction has some impact on DNA repair or chromatin remodelling? (3) How does FKBP25 cause auto-ubiquitination of MDM2? (4) Can FKBP25 recognize other secondary structures of DNA like G-quadruplex DNA? These studies will help us better understand the biological significance of FKBP25, a very unique member of the FKBP family, in gene regulation, DNA repair, chromatin remodelling and also for further downstream clinical studies in the arena of immunosuppression.

Author's Publications

- 1) **Ajit Prakash.**, Shin J. and Yoon H.S. (2015) H, C and N resonance assignments of human FK506 binding protein 25. *Biomol NMR Assign*, **9**, 43-46.
- 2) **Ajit Prakash.**, Joon Shin., Sreekanth Rajan., and Ho Sup Yoon. (2016) Structural basis of nucleic acid recognition by FK506-binding protein 25 (FKBP25), a nuclear immunophilin. *Nucleic Acids Research*. *doi: 10.1093/nar/gkw001*.
- 3) **Ajit Prakash.**, Sreekanth Rajan., and Ho Sup Yoon.(2016) Crystal structure of the FK506 binding domain (FKBD) of human FKBP25 in complex with FK506 drug. *Protein Science*. *doi:10.1002/pro.2875*.
- 4) **Ajit Prakash.**, Anjali S., Phan A.T., and Yoon H.S. Human FKBP25, a novel G-quadruplex binding protein. (Manuscript under preparation for **Journal of the American Chemical Society**)
- 5) **Ajit Prakash.**, Shin J. and Yoon H.S. Characterization of histone deacetylase inhibitors apicidin and trapoxin as FKBP25 binding drugs. (Manuscript under preparation for **Journal of the American Chemical Society**)

Conference papers

1. Structural and molecular studies of FKBP25-DNA interaction. Conference conducted by VIB Belgium from 9-10 February.
2. Structural basis of interaction of FKBP25 with double stranded DNA and G-quadruplex DNA. EMBO workshop conducted by NISB, NTU Singapore from 7-10 December.

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Appendix

Appendix 1: Assignments of all the residues of FKBP25

Residue	Chemical shift of N	Chemical shift of H
A3N-H	124.648	8.139
A4N-H	124.655	8.086
V5N-H	120.991	7.766
Q7N-H	118.919	8.028
R8N-H	125.445	8.215
A9N-H	129.103	7.118
W10N-H	115.651	6.973
T11N-H	115.239	8.711
V12N-H	121.12	8.765
E13N-H	119.089	8.259
Q14N-H	119.504	7.462
L15N-H	119.046	7.985
R16N-H	114.226	7.496
S17N-H	116.542	7.102
E18N-H	125.062	8.794
Q19N-H	116.601	7.896
L20N-H	123.927	6.971
K22N-H	122.069	8.688
K23N-H	116.086	8.764
D24N-H	121.205	7.087
I25N-H	121.417	7.059
I26N-H	119.621	8.222
K27N-H	120.445	8.368
F28N-H	118.84	7.519
L29N-H	120.364	8.55
Q30N-H	117.357	8.932
E31N-H	117.71	7.781
H32N-H	112.656	7.193
G33N-H	110.529	8.775
S34N-H	118.51	8.119
D35N-H	122.997	8.862
S36N-H	115.453	8.421
F37N-H	127.69	7.701
A39N-H	123.035	8.389
E40N-H	120.799	7.877
H41N-H	114.382	6.94

K42N-H	117.813	7.655
L43N-H	116.595	8.183
L44N-H	119.6	7.239
G45N-H	110.585	8.17
I47N-H	127.744	8.775
R48N-H	119.842	8.042
N49N-H	117.938	7.485
V50N-H	122.272	8.184
A51N-H	120.114	8.321
K52N-H	114.853	7.061
T53N-H	107.972	7.264
A54N-H	126.518	7.681
N55N-H	120.445	8.368
D57N-H	119.255	8.199
H58N-H	120.799	7.877
L59N-H	119.193	7.739
V60N-H	121.728	8.547
T61N-H	116.47	7.708
A62N-H	123.847	8.085
Y63N-H	120.725	8.654
N64N-H	116.857	8.461
H65N-H	120.792	8.77
L66N-H	124.679	8.448
F67N-H	113.836	6.79
E68N-H	120.538	8.236
T69N-H	106.8	7.742
K70N-H	118.261	7.518
R71N-H	122.505	6.126
F72N-H	118.322	6.98
K73N-H	123.894	8.006
T75N-H	111.522	7.721
E76N-H	123.124	8.351
S77N-H	117.86	8.29
I78N-H	123.245	8.033
K80N-H	124.685	8.223
V81N-H	122.071	8.013
S82N-H	119.949	8.203
E83N-H	123.613	8.435
Q84N-H	121.925	8.195
V85N-H	122.264	7.97
K86N-H	125.414	8.178

V88N-H	121.527	8.454
L90N-H	124.648	8.139
N91N-H	119.871	8.286
K94N-H	123.231	7.971
K96N-H	122.927	8.322
T98N-H	117.244	8.134
K99N-H	125.289	8.301
S100N-H	118.917	8.317
E101N-H	123.724	8.249
E102N-H	122.493	8.259
T103N-H	116.968	8.099
L104N-H	126.081	8.205
D105N-H	122.836	8.231
E106N-H	121.925	8.195
G107N-H	110.673	8.161
Y111N-H	112.469	6.708
T112N-H	113.172	8.669
K113N-H	126.269	8.989
S114N-H	123.464	8.961
V115N-H	128.437	9.114
L116N-H	130.675	9.025
K117N-H	121.652	7.972
K118N-H	126.641	8.588
G119N-H	111.559	8.639
D120N-H	118.148	8.142
K121N-H	116.662	8.701
T122N-H	115.399	8.883
N123N-H	125.801	9.667
F124N-H	123.316	8.289
K126N-H	122.051	9.009
K127N-H	119.793	8.336
G128N-H	115.522	9.076
V130N-H	121.421	7.908
V131N-H	117.99	7.859
H132N-H	117.872	7.209
C133N-H	118.551	9.42
W134N-H	124.753	8.561
Y135N-H	122.02	9.712
T136N-H	116.455	8.433
G137N-H	118.721	9.197
T138N-H	118.359	9.26

L139N-H	120.565	8.511
Q140N-H	122.353	9.541
D141N-H	116.451	7.579
G142N-H	109.004	8.027
V144N-H	129.775	8.691
F145N-H	126.397	8.046
D146N-H	120.035	6.572
T147N-H	120.422	7.96
N148N-H	123.871	7.552
I149N-H	122.583	8.155
Q150N-H	126.71	8.108
K156N-H	120.215	7.614
K157N-H	122.102	8.1
A159N-H	124.543	7.681
K160N-H	122.287	8.361
L162N-H	124.023	7.897
F164N-H	119.111	7.564
K165N-H	121.196	8.196
V166N-H	127.06	8.827
G167N-H	116.444	9.5
V168N-H	111.865	8.167
G169N-H	114.393	8.817
K170N-H	121.433	9.426
V171N-H	108.275	6.372
I172N-H	111.709	7.239
R173N-H	125.247	8.379
G174N-H	132.95	9.31
W175N-H	119.592	6.822
D176N-H	121.479	7.535
E177N-H	111.73	8.053
A178N-H	121.636	7.228
L179N-H	120.492	8.028
L180N-H	112.544	6.614
T181N-H	106.76	7.698
M182N-H	126.389	7.557
M182N-H	125.835	8.21
S183N-H	110.755	7.206
K184N-H	121.528	7.879
G185N-H	118.173	8.757
E186N-H	124.207	8.534
K187N-H	124.704	8.709

A188N-H	129.763	9.424
R189N-H	122.293	9.147
L190N-H	128.905	9.927
E191N-H	124.056	8.567
I192N-H	127.005	9.552
E193N-H	126.054	8.149
E195N-H	118.394	9.54
W196N-H	120.394	7.89
A197N-H	125.345	7.872
Y198N-H	122.84	9.384
G199N-H	108.841	8.342
G202N-H	103.727	7.014
A206N-H	122.312	7.379
K207N-H	112.292	7.5
N211N-H	118.096	8.119
A212N-H	121.651	7.665
K213N-H	126.286	8.337
L214N-H	126.286	8.337
T215N-H	119.928	8.525
F216N-H	124.631	9.59
E217N-H	123.367	8.764
V218N-H	123.942	8.968
E219N-H	129.099	9.241
L220N-H	130.846	8.666
V221N-H	127.096	8.717
D222N-H	114.584	7.441
I223N-H	121.293	8.675
D224N-H	133.714	9.011

Appendix 2: Chemical shift deviation of $H\alpha$, ^{13}Ca , $^{13}C\beta$ and ^{13}CO of FKBP25 from RC value.

Figure: The chemical shift deviation from random coil values of $H\alpha$, ^{13}Ca , $^{13}C\beta$ and ^{13}CO plotted for all the residues of FKBP25. The values of HA, CO, CA and CB are shown in blue, red, green and purple colored bars. Chemical shift deviation were calculated in ppm.

Appendix: 3: A symmetric NOE of Q7 on the carbon plane of K187

Figure: Representative strips of the ^{13}C -edited three-dimensional NOESY spectrum of FKBP25, with NOE cross-peaks between residues on the N-terminal HLH Gln7 and the C-terminal FKBD Lys187.

Appendix 4: Plots of the ^{15}N Relaxation data of free FKBP25

Figure: R_1 (top), R_2 (second), ^1H - ^{15}N heteronuclear NOE values (third) and R_2/R_1 ratio (bottom) measured at 298 K on a 700 MHz NMR spectrometer. N-terminal HLH, C-terminal FKBD (rectangular) and flexible internal linker are shown on top of the panel.