学位論文

Analysis of nuclear FK506-binding protein (FKBP)

核に局在するFK506結合蛋白質の解析

—単離からクロマチンへの作用の解析—

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東京大学大学院薬学系研究科

生命薬学専攻

葛原　隆

Contents

Prologue

Our world is a three-dimensional space. Thus, all molecules, including proteins, have three-dimensional structures. We change as time passes. Thus, the structures of all molecules also change with time. Therefore, my ultimate goal is to elucidate how biological molecules change their structure.

Foreword

1. Molecular interactions and chemical reactions

All life can be explained in terms of physical and chemical machines, so it is important to understand the mechanisms of biological reactions from physical and chemical perspectives. Biological reactions are broadly classified into "molecular interactions" and "chemical reactions".

A "molecular interaction" is a non-covalent association of two or more molecules, that induces conformational changes in those molecules and in subsequent reactions. Their importance is explained by the following examples: Interactions between DNA-binding domains and DNA have been reported to be essential for all nuclear reactions on DNA, including transcription, replication, repair and recombination. Similarly, interactions between transcriptional activation domains and transcriptional machinery are known to play central roles in transcriptional activation. In signaling cascades, G proteins, kinases and other signal proteins associate with cytosolic domains of receptors and activate downstream signals. Various extracellular ligands associate with

receptors on the cell surface and activate them. Cell-cell interactions through cell adhesion molecules are related to growth and differentiation. These phenomena are the basis of life.

"Chemical reactions" include all enzymatic reactions. Examples include (but are not restricted to), synthesis, degradation and metabolism of nucleic acids, protein, lipid, sugar and other biological molecules; modification (acetylation, methylation, phosphorylation, ubiquitination etc.) of histones and other proteins; the TCA cycle; and the electron transfer system. The importance of these reactions for life is beyond dispute.

2. Conformational changes

Both molecular interactions and chemical reactions induce conformational changes of proteins. For example, the general transcription factor TFIID has been reported to interact with transcriptional activators and to change its conformation on the promoter DNA (1-3). These conformational changes induce sequential molecular interactions and chemical reactions. Conformational change of proteins is purported to serve as a "molecular switch" for initiating a cascade of molecular reactions. Therefore, it is imperative to investigate the mechanisms underlying protein conformational changes. This phenomenon is mainly mediated by the rotation of the major peptide chains via one of two mechanisms. Firstly, rotation of the bond between C alpha and C or N in the peptide bond occurs in all amino acids in their hinges or loops and is a continuous form of rotation. Alternatively, cis/trans isomerization of the peptidyl prolyl imide-bond can occur, this is a discontinuous rotation event with only the only two opposing

directions permitted (4). Thus, in my opinion this latter mechanism is well suited to serving as a switching mechanism between "ON" and "OFF" in biological reactions.

Many biological molecules form complexes *in vivo*. Thus, structural change in the formation of macromolecular complexes (protein, DNA, RNA, sugar, lipid etc.) is also pivotal for biological reactions. Structural changes of complexes include variations in the association and dissociation of their components. Thus, the associations and dissociations of macromolecular complexes are the central theme of this thesis.

3. Alteration of chromatin structure

DNA, which codes genetic information, forms condensed structures in cells – termed "chromatin" – with histones and other chromatin proteins. Various nuclear reactions, including DNA transcription, replication, repair and recombination, induce dynamic alteration in chromatin structures. In the alteration of chromatin structure, the structure of the components of chromatin, such as DNA and protein, must undergo change. In nuclear reactions, "ON" and "OFF" must be clearly distinguished. On the basis of these combined observations, I speculated that PPIase should play a role in the alteration of chromatin. Therefore, I have started to investigate the importance of nuclear PPIases for chromatin organization. (Introduction figures 1 & 2)

To discover a novel mechanism of gene expression, it is essential to identify previously unknown factors, domains or enzymes. Here, I report that nuclear FKBP (FK506-binding protein), one of the PPIases, is a novel chromatin-modulating factor. I

demonstrate that nuclear FKBP is a dual functional protein comprising histone chaperone and PPIase activities. I also show that nuclear FKBP participates in rDNA silencing, which is involved in aging. These findings shed new light on chromatin analysis and suggest a link between alteration of nucleosome structure and isomerization of prolyl bonds. (Introduction figures 1 & 2)

4. Thesis outline

This thesis describes how nuclear FK506 binding protein (FKBP) has two distinct activities that mediate structural changes.

In the 1st section, I will report the isolation and characterization of a new nuclear FKBP. The *Schizosaccharomyces pombe* (*S.pombe*) gene, *fkp39+*, that encodes a homologue of FKBP (FK506 binding protein)-type peptidyl prolyl *cis/trans* isomerase (PPIase), was isolated and its primary structure was determined. This gene product (SpFkbp39p) shows PPIase enzymatic activity in a chymotrypsin-dependent enzyme assay. Comparison of the primary structures of catalytic domains of FKBPs, including SpFkbp39p, indicated that FKBPs could be classified mainly into four groups. Here, I report that this categorization corresponds to known subcellular localizations of FKBPs, suggesting that the subcellular localization of FKBPs may be predicted from their primary structures. On the basis of this categorization, SpFkbp39p was suggested to be a member of the nuclear-type FKBP group. Subcellular localization of HA-epitope-tagged SpFkbp39p by immunofluoresence assay indicated that SpFkbp39p is localized to the nucleus, in agreement with my prediction. I also identified residues which are conserved in a "group-specific" manner in the catalytic domain, mapped them to

corresponding three-dimensional positions and found that several "group-specific" residues are closely aligned in distinct regions, mainly on the protein surface, thereby implying the presence of "group-specific" regulatory functional regions. I also found that nuclear-type FKBPs, including SpFkbp39p, possess two highly conserved domains other than the catalytic domains, in addition to basic and acidic charged regions. These characteristics are specific for nuclear-type FKBPs.

In the 2nd section, I demonstrate the activities of nuclear FKBP towards chromatin. Alteration of chromatin structure is controlled by the concerted actions of several sets of enzymes and factors. These proteins include histone chaperones, ATP-dependent chromatin remodeling factors, and enzymes that covalently modify chromatin proteins, such as histone acetyltransferases and deacetylases. Here, I describe that a nuclear FK506 binding protein (FKBP), one of the peptidyl prolyl *cis/trans* isomerases (PPIases) originally identified as enzymes that assist in the proper folding of polypeptides, possesses histone chaperone activity. This histone chaperone activity resides in the N-terminal region of the protein and is clearly distinct from the C-terminal PPIase domain. Analyses of null mutants of nuclear FKBP genes indicate that endogenous nuclear FKBPs are required to regulate silencing of gene expression and alteration of the chromatin structure at the rDNA locus. The N-terminal domain alone can function in chromatin organization in yeast cells. These *in vitro* and *in vivo* observations demonstrate that nuclear FKBPs represent a novel type of chromatin modulating factor.

5. Brief overview of PPIases

1. Identification of PPIases

Folded proteins contain cis-prolines while newly translated proteins only contain trans-prolines. This suggests the existence of an enzyme that isomerizes the cis/trans proline transition. Based on this prediction, enzymes which catalyze this reaction have been identified and purified. Determination of the primary structure of these enzymes (peptidyl prolyl cis/trans isomerase (PPIase)) demonstrated that PPIases are the same enzymes as immunophilins (cyclophilin and FKBP). (Introduction figure 1)

2. PPIase families

PPIases are classified into three families on the basis of their primary structures and known inhibitors. The FKBP family binds to FK506, whereas the cyclophilin family binds to cyclosporin A and PIN1 does not bind to either. Thus, the specificities of these PPI families are distinct from each other.

3. Relevance of focusing on PPIases

I here isolated chromatin factors by a two-hybrid system using general transcription factors as baits. FKBP was isolated because it interacted with CCG1, the largest

subunit of TFIID. Since both the structures of FKBP and TFIID are highly conserved, it is reasonable that they should interact with each other. This prompted me to study the influence of FKBP on chromatin factors.

Chapter One

Isolation and initial analyses of nuclear FKBP-type PPIases

1.1 Introduction

Conformational changes of proteins frequently affect protein-protein interactions, which play crucial roles in controlling the qualitative state of protein function. The peptidyl prolyl cis-trans isomerase (PPIase) may induce a conformational change in proteins in vivo because it catalyzes interconversion of cis- and trans- isomers of peptidyl-prolyl bonds in peptide and protein substrates in vitro (1, 2). PPIase is present in both prokaryotes and eukaryotes and primary structures of their catalytic domains are highly conserved (3). PPIases are thought to be involved in protein folding (4) because these enzymes accelerate the refolding of denatured proteins in vitro (5-7). On the other hand, interactions of PPIases with specific molecules suggest that PPIases may have other functions. For example, FK506 binding proteins (FKBPs), submembers of the PPIase family, have been shown to interact with various factors (e.g. FKBP12 interacts with TGF_ type I receptor (8) and transcription factor YY1 (9), FKBP52 (also known as p59, FKBP59, HSP56 or HBI) with steroid receptors (10-12), FKBP25 with casein kinase II and nucleolin (13)). Interactions between FKBPs and specific target molecules affect the functional state of their targets, in

several cases (9, 14). Recent genetic analysis using multi-cellular organisms revealed that FKBPs is involved in biological phenomena such as development (15-17).

PPIases can be classified into three subfamilies on the basis of differences in sensitivity to potent inhibitors, the FKBP, cyclophilin (CyP) and parvulin families, respectively. FK-506 and cyclosporin A selectively bind FKBPs and CyPs, respectively, and inhibit PPIase activities (18-21), whereas the parvulin family is insensitive to these drugs (22). The primary structures of the catalytic domains are similar to each other within the subfamily members but differ among the three subfamilies. Moreover, comparisons of the tertiary structure of each protein revealed that similar residues among primary structures in each subfamily are also limited by a three-dimensional structure (23). In eukaryotic cells, PPIases are localized in various cellular compartments (21). In S.cerevisiae, there are four FKBPs (24-29), eight cyclophilins (29-36) and one parvulin (22, 37), and that three PPIases are localized in the cytoplasm (Fpr1, Cpr1, 6), four in the ER (Fpr2, Cpr2, 4, 5), one in the mitochondria (Cpr3) and two in the nucleus (Fpr3, Ess1) (22, 29), suggesting that functional targets of PPIase might be diversified.

To elucidate functional roles of PPIase in each cellular compartment, but particularly in the nucleus, we isolated a nuclear-type PPIase from S.pombe, a favorable model organism to analyze biological function both biochemically and genetically. Clues to understanding the universal molecular mechanisms between mono- and multi-cellular organisms may be found in S.pombe because molecular mechanisms of the several biological phenomena in S.pombe are more analogous to those in multicellular organisms than those in S.cerevisiae, in several aspects (38, 39). The evolutionary

distances among S.pombe, S.cerevisiae and multicellular organisms also make it useful to identify potent functional motifs by comparing structures among their homologues.

We now report the primary structure of a novel gene, Spfkp39+ which was isolated as a FKBP-type PPIase in S.pombe. Comparative studies using primary structures of FKBPs indicate that FKBPs can be classified into four groups on the basis of differences in primary structures of catalytic domains. This may allow one to predict subcellular localization of FKBPs from primary structures, not of localization signals, but of catalytic domains. We also determined several biochemical characteristics of SpFkbp39p by showing nuclear localization as well as identifying several novel structural motifs, which are specific for the nuclear-type FKBP group.

1.2 Experimental procedures

1.2.1 Isolation of a S.pombe DNA fragment containing fkp39+ by PCR using degenerate oligonucleotide primers

General methods for DNA manipulation were as described (40). Two degenerate oligonucleotide PCR primers (5'-SAR GTI ATH MRI GSI TGG-3' and 5'-ARY TCI ACI TCR AAI RYI ARI GT-3'. I, inosine; S, cytosine or guanine; R, adenine or guanine; H, adenine, thymine or cytosine; M, adenine or cytosine; W, adenine or thymine; Y, cytosine or thymine) designed from the sequences, E/Q-V-I-K/R-A/G-W and T-L-V/T-F-E/D-V-E-L, of the catalytic domains conserved among FKBPs, were used to amplify DNA fragments from S.pombe genomic DNA by Polymerase Chain Reaction (PCR). PCR amplification was performed using Taq DNA polymerase (Boehringer Mannheim), manufacturer's buffers and GeneAmp PCR System 9600 (Perkin Elmer). The PCR protocol cycles was for 30 sec at 95 , 2 min at 40 and 1 min at 72 . The resulting PCR products were subcloned into pBluescriptIISK(-) (Stratagene) at the SmaI site and the nucleotide sequences were determined using Dye Terminator Cycle Sequencing Ready Reaction DNA sequencing kits (Perkin Elmer) and a DNA sequencer ABI377 (Perkin Elmer), according to the manufacturers' instructions.

1.2.2 Isolation of S.pombe genomic DNA fragments containing full-length fkp39+

To isolate the full-length open reading frame, PCR-amplified DNA fragments were used to screen the S.pombe genomic library (provided by Dr. A. Ishihama) by plaque hybridization, using the Gene Images random prime labeling and detection system (Amersham), according to the manufactures' instructions. The three independent positive clones isolated were digested with ApaI and XhoI and subcloned into pBluescriptIISK(-) (Stratagene) all at the same sites. This plasmid was named pBS-Spfkp39+. Nucleotide sequences of these clones were determined on both strands, as described above. This nucleotide sequence has been submitted to the GenBank ⁊ with accession number AF017990.

1.2.3 Construction of plasmids which express Fkbp39p protein

BamHI and NdeI sites were engineered at the translation initiation site and BamHI site at the translation stop codon of SpFkbp39p by PCR. Two primers 5'-GCG GAT CCA TAT GTC TCT TCC AAT TGC TG-3', which has BamHI and NdeI sites, and 5'-CCG GAT CCT TAG TGA ACG CGA ACA AGC TTG ACT TC-3', which has a BamHI site, were used to amplify the fragment from pBS-Spfkp39+ plasmid. This fragment was subcloned into pBluescriptIISK(-) (Stratagene) at BamHI sites, as described above. This vector was named pBS-Fkbp39p. pBS-Fkbp39p was digested at NdeI and BamHI sites and was subcloned into 6His-pET11d (41) and pREP1HA2 (T. Kuzuhara

and M. Horikoshi, unpublished results), a pREP1 (42) -based S.pombe vector which expresses proteins with two copies of the influenza virus hemagglutinin (HA) sequence (YPYDVPDYA) appended to its NH2-termini. The resultant plasmids were named pET11d-6His-Fkbp39p and pREP1-HA-Fkbp39p, respectively.

1.2.4 [3H]dihydro-FK506 binding assay

Binding of [3H]dihydro-FK506 (NEN) was determined using the LH-20 assay (25) with 12nM [3H]dihydro-FK506 and 0.1 μM SpFKBP39p in 20mM Tris, pH7.5, 200mM NaCl. Bound [3H]dihydro-FK506 was separated from free [3H]dihydro-FK506 by chromatography on individual Sephadex LH-20 columns (Pharmacia). Samples were fractionated and counted in an LS-6000LL scintillation counter (Beckman instruments).

1.2.5 Determination of subcellular localization of SpFkbp39p

The plasmid pREP1-HA-Fkbp39p, pREP1-HA-TBP (T. Yamamoto and M. Horikoshi, unpublished results) and mock plasmid pREP1 were transformed into S.pombe strain JY741 (h- ade6-M216 leu1 ura4-D18), using general methods. Staining of these yeast cells by indirect immunofluorescence was done essentially as described (44) but with the following modification. Cells were cultured in the 4ml of adenine and uracil

complemented MM medium (MMAU) to 106cells/ml. Cell walls were removed by incubation with 20 ｵg/ml Zymolyase-20T (Wako) at 30 for 30 min. Primary antibodies (anti-HA mouse IgG (KODAC)) were incubated with the fixed cells at room temperature for over 12hr. Secondary antibodies (FITC-labeled anti-mouse IgG rabbit IgG (Wako)) were applied for over 12 hr. Cells were stained with Hoechst 33342 (SIGMA), examined microscopically and photographed (Fuji color slides).

1.2.6 Peptidyl-prolyl cis/trans-isomerization activity of recombinant FKBPs

PPIase activity was assayed essentially as described (1, 43). The assay measures the ﾂ cis ﾂ to ﾂ trans ﾂ isomerization of the proline-leucine peptide bond in the peptide, N-succinyl-Ala-Leu-Pro-Phe-p-nitroanilide (Bachem). The release of p-nitroanilide is quantitated spectrophotometrically at 390 nm. Reactions (1ml) were at 0 and contained 0.17mM substrate, 50 mM Hepes-Na (pH 8.0), 100mM NaCl, FKBPs and 10_M FK506 (Fujisawa Pharmaceutical Co., Ltd.). Immediately before start of the assay, 10 ｵl of chymotrypsin solution (10mg/ml in 0.001 M HCl; final concentration 1mg/ml) was added. After mixing, the increase in absorbance at 390nm was measured in a Beckman DU680 spectrophotometer at 1 s intervals (Fig. 3). The first order rate constant, k (s-1), was calculated from the slope of the resulting line (Table 1).

1.2.7 Method of computer analysis

CLUSTAL W (46) was used for the alignments of amino acid sequences and NJPLOT for the determination of the phylogenetic tree. The three-dimensional structure of HsFKBP12 was drawn and analyzed with RasMac (ver. 2.5). Other analyses were performed by GENETYX-MAC (ver. 9.0) (SOFTWARE DEVELOPMENT CO.,LTD.).

1.3 Results

1.3.1 Isolation of fpk39+ gene

To isolate novel S.pombe nuclear-type PPIase genes, PCR was carried out using degenerate oligonucleotide primers based on the conserved catalytic domains of FKBPs. The amplified S.pombe genomic DNA fragments were isolated and nucleotide sequences determined. The deduced amino acid sequence encoded a fragment (arrow under the nucleotide sequence (1525 - 1665) in Fig. 1) showing high similarity (over 40% identity) to the catalytic domains of other FKBPs. To obtain the full-length clone, we screened a S.pombe genomic library, using this PCR fragment as a probe. Three independent positive clones were isolated and nucleotide sequences were determined.

An open reading frame (ORF) is located from 601 (start codon: 601 - 603) to 1685 (stop codon: 1686 - 1688) (Fig. 1). An in-frame stop codon locates six bases upstream (position 595 - 597, underlined in Fig. 1) of the putative translation initiation site. There are no consensus sequences for donor, acceptor or branch sites for splicing in this region (46). The deduced amino acid sequence of this ORF is most like that of S.cerevisiae FKBP-type PPIase, Fpr3 (45% identity) (27, 28), consisting of 361 amino acids (39,301 daltons) with the FKBP catalytic domain located at the C terminal region (267 - 361, double underlined in Fig. 1). Two putative nuclear targeting sequences which consist of bipartite short stretches of basic amino acids with non-conserved linker regions (47) located in positions 184 - 209 and 215 - 239 (boxed (basic stretch) and

dotted-lined (linker) in Fig. 1), suggesting nuclear localization. Taken together with the results shown above, we conclude that this isolated gene encodes a putative nuclear-type FKBP, named SpFkbp39p, most likely to be the S.pombe homolog of S.cerevisiae Fpr3.

1.3.2 SpFkbp39p binds to FK506 and has PPIase catalytic activity

The primary structure of the COOH-terminal region of SpFkbp39p is similar to those found in catalytic domains of FKBPs. FKBP-type PPIases interact with FK506 and their PPIase enzymatic activities are inhibited by these interactions. To determine if SpFkbp39p has biochemical characteristics of FKBP-type PPIases, we prepared bacterial-expressed SpFkbp39p protein. The plasmid pET11d-6His-Fkbp39p, which expresses His-tagged SpFkbp39p in Escherichia coli, was constructed and recombinant His-tagged SpFkbp39p (His-SpFkbp39p) was purified by Ni-agarose chromatography as described in Experimental Procedures.

We first observed the interaction between SpFkbp39p and FK506. Purified recombinant His-SpFkbp39p bound [3H]dihydro-FK506, which was detected by the LH-20 assay (25) (Fig. 2B). Next, we measured PPIase catalytic activity of SpFkbp39p by enhancement of the rate of chymotrypsin cleavage of a synthetic Pro-containing peptide substrate in a coupled spectrophotometric assay (1, 43). This assay demonstrated that His-SpFkbp39p protein has PPIase activity comparable to HsFKBP12

(control), and that this enzymatic activity is also almost perfectly inhibited by the FK506 (Fig. 3). The specific activities (kc/Km) of these molecules in reactions are summarized in Table 1, which shows that the kc/Km of control enzyme, HsFKBP12 is similar to that of previous data (1, 43), and that the kc/Km of SpFkbp39p is almost half that of HsFKBP12. Taken together, the results obtained from these biochemical assays indicate that SpFkbp39p has FKBP-type PPIase activity in vitro.

1.3.3 Classification of FKBPs by primary structure of catalytic domains

Distinct FKBPs are localized to different cellular compartments (21). It is speculated that differences in the primary structures of catalytic domains reflect differences in subcellular localization of FKBPs because the substrates and/or interactors of FKBPs might differ in each cellular compartment. Therefore, we compared the primary structure of SpFkbp39p with eukaryotic FKBPs in order to elucidate the relationship between the primary structure and subcellular localization of FKBPs.

Phylogenetic tree analysis based on the primary structures of catalytic domains of known FKBPs (Fig. 4) indicated that 40 FKBPs can be classified mainly into four groups (group A to D), excluding only four FKBPs. Surprisingly, this classification corresponds to categorization by subcellular localization of each group; localized in nucleus (group A), cytoplasm (group B), nucleus or nucleus and cytoplasm (group C) and ER (group D). This observation may make feasible prediction of the

subcellular localization of FKBPs from primary sequences of catalytic domains.

1.3.4 Detailed analysis of amino acid residues of catalytic domains

FKBPs are classified into four groups by primary structure, as shown in Fig. 4. To examine whether this classification of FKBPs reflects differences in amino acid residues among each group protein, we mapped the "conserved" and "group-specific" residues to alignment of the primary structures of FKBPs (Fig. 5A). We defined the amino acid residues with over 90% identity and 100% similarity in all groups as "conserved" residues (blue- and red-, and cyan shadowed, respectively, in Fig. 5A), and the amino acid residues which are completely identical and similar within one, two or three group(s) except "conserved" residues as "group-specific" residues (green-shadowed in Fig. 5B). The alignment shows that both "conserved" and "group-specific" residues are not closely located to a part but rather located to catalytic domains in a non-consecutive manner. However, it is possible that these residues might form functional regions and thus be located together, at the three-dimensional level.

To define the presence of the putative functional domains discussed above, we mapped these conserved and group-specific residues to these corresponding positions in the three-dimensional structure of human FKBP12 (HsFKBP12) the tertiary structure of which has been determined (48, 49) (Fig. 5B and Fig. 5C). The similarity of primary structures and tertiary structures (FKBP12 (group B) (48, 49), FKBP52 (group C2) (50)

and FKBP25 (group C1) (J. Liang, D. T. Hung, S. L. Schreiber and J. Clardy, unpublished results shown in PDB database)) of catalytic domains of FKBPs suggests that the backbone structure of the catalytic domains of most FKBPs should be similar as well. As a result, the "conserved" residues (blue, red or cyan in Fig. 5) are located in proximity (Fig. 5B) and some form a hydrophobic pocket (48, 49) which is required for FKBP - immunoretardant interactions and basal PPIase activity. Interestingly, "group-specific" residues (green in Fig. 5) are also located together in several regions and sites which have not been described (circled in Fig. 5B and 5C) although these residues are located in a non-consecutive manner in primary structure. Because these regions and sites consist of group-specific residues which exist in FKBPs in a group-specific manner, they might be involved in "group-specific" functions and for which our classification of FKBPs by phylogenetic tree based on primary structure of catalytic domains can be appropriate.

Several FKBPs interact with specific molecules (9-11, 13, 14). It would be of interest to investigate whether "group-specific" regions, as shown above, are required for interactions between FKBPs and known interactors. If so, this will lead to elucidation of the "group-specific" function of FKBPs.

1.3.5 Nuclear localization of SpFkbp39p

We proposed the presence of four FKBP groups and group-specific regions. From this

proposal, SpFkbp39p is predicted to localize to the nucleus. This provides experimental support of our hypothesis on the relationships between primary structure of catalytic domains of FKBPs and their subcellular localization. We analyzed the subcellular localization of SpFkbp39p, using the fluorescence antibody technique to elucidate localization in the cell. We expressed HA-tagged SpFkbp39p and TBP (TATA box-binding protein) in S.pombe cells and an anti-HA antibody was used for detection.

This analysis revealed that the nucleus (stained with Hoechst 33342) of the resultant cells carrying pREP1-HA-SpFkbp39p was stained with FITC, similar to the control cells carrying pREP1-HA-TBP (Fig. 6), which indicates that SpFkbp39p is localized to the nucleus. This observation supports the validity of our prediction on subcellular localization of the FKBP family. Further investigations are expected to define rules on subcellular localization of FKBPs.

1.4 Discussion

1.4.1 Characteristics of group A FKBPs

The newly isolated SpFkbp39p is classified into nuclear-type group A with five other known FKBPs, and SpFkbp39p is localized to the nucleus (Fig.6). To identify conserved structural motifs other than the catalytic domain which might act as a functional domain, we compared the full-length primary structures of group A FKBP with members of other group FKBPs. Interestingly, group A proteins have long NH2-terminal regions adjacent to the catalytic domains (Fig. 7B). Alignment among group A proteins in these regions demonstrates that they have two novel conserved domain structures specific for group A FKBPs which were named conserved domains I (15% - 41% identity and 41% - 70% similarity) and II (30% - 55% identity and 50% - 70% similarity), as shown by the colored-box in Fig. 7A. They also contain acidic (in SpFkbp39p, underlined in Fig. 1, and red-lined above in Fig. 7A) and basic (bold in Fig. 1, and blue-lined above in Fig. 7A) regions (26, 27, 51, 52) which are also rich in both Ser and Thr residues (Fig. 7A). Their orders of each domain and region are the same (Fig. 7B), suggesting that not only the primary structure but also positions of each domain and region might be significant for the functional roles of group A proteins. This domain structure is not present in other groups (ScFpr1 (group B), HsFKBP52 (group C1), HsFKBP25 (group C2) and ScFpr2 (group D)) (Fig. 7B), and strongly suggests that group A FKBPs form a functional subfamily. The primary structures of

yeast and insect group A FKBPs differ in the NH2-terminal regions. Therefore, we designated this a "species-specific" region (Fig. 7B), suggesting that there is functional diversity among group A.

1.4.2 Conclusion and perspective

In conclusion, we isolated a novel S.pombe gene product, SpFkbp39p, which forms a nuclear-type subfamily with several FKBPs. This subfamily is classified according to differences in the primary structures of FKBP catalytic domains. SpFkbp39p has biochemical properties similar to those of other FKBPs. Moreover, we identified novel domain structures conserved in the N-terminal regions found only among nuclear-type FKBPs. These structural motifs include both negatively- and positively-charged regions which might interact with positively-charged proteins in the nucleus, such as histones, and acidic molecules, such as DNA, respectively.

Chapter Two

A nuclear FK506-binding protein (FKBP) has a histone chaperone activity and regulates rDNA silencing

2.1 Introduction

Alterations in chromatin structure are important in regulating the access to DNA of the complex machinery that controls nuclear phenomena such as gene expression (1). Changes in chromatin structure are effected by both enzymatic and non-enzymatic activities (2); examples of the latter include the actions of histone chaperones (3-5). These proteins mediate nucleosome assembly and disassembly, and these dynamic alterations in nucleosome structure in turn influence nuclear events (3-5). Histone chaperones function in concert with enzymes such as chromatin-remodeling factor and chromatin-modifying enzyme to stimulate or inhibit processes such as DNA replication and transcription (4,5).

Peptidyl prolyl cis/trans isomerase (PPIase) enzymes alter the orientation of peptide chains at proline residues, thereby aiding proper protein folding (6). PPIases have been classified into three subfamilies (FKBP, Cyp and Pin1) and are reported to be localized in the nucleus and/or cytosol (7). Nuclear FKBP proteins possess other

elements in addition to the PPIase domain (8). In addition to the diversity of PPIases with respect to their structure and localization, recent data indicate that PPIases function by associating with other proteins, thereby modulating their activities (6,7). For example, in the nucleus, the DNA-binding activity of the c-Myb transcription factor is negatively regulated by its stable interaction with Cyp40 (9). In addition, FKBP52 inhibits the DNA-binding and transactivation properties of the transcription factor IRF-4 (10). Moreover, the multi-domain PPIases FKBP51 and/or Cyp associate with steroid receptors in heterocomplexes to modulate receptor activity (11-13) via their additional domains in vitro. Furthermore, the intranuclear prolactin/cyclophilin B complex acts as a transcriptional inducer (14), and another nuclear PPIase, Pin1, regulates a subset of mitotic phosphoproteins and restores the function of the Alzheimer-associated phosphorylated tau protein (15). Pin1 also interacts with the Cdk9-phosphorylated hSpt5 subunit of the transcription elongation factor DSIF (16), and generates conformational changes in p53 that enhance its transactivation activity (17-19). Collectively, these results suggest that PPIases regulate protein activity by controlling the assembly and/or disassembly of multiple protein complexes (7).

We speculated that nuclear PPIases may contribute to the dynamics of chromatin complex assembly and/or disassembly because they have been reported to interact with basic chromatin components. For example, the S. cerevisiae nuclear PPIase Fpr3 binds to a histone H2B nuclear localization signal (20). Moreover, Essl, a yeast homologue of Pin1, was shown to be genetically linked to histone deacetylase Rpd3 (21,22). In addition, FKBP25 has been reported to associate with histone deacetylase

(23). However, it has not been tested whether PPIases themselves can alter chromatin structure.

We previously isolated a nuclear FKBP from S. pombe, termed SpFkbp39p, which has a charge-rich domain and two conserved domains in its N-terminus, in addition to a C-terminal PPIase domain (8). We predicted that SpFkbp39p could interact with histones and DNA (8). In this study, we show that nuclear FKBP has dual functions, namely, histone chaperone activity and PPIase activity, and that it is required for silencing at the rDNA locus. Furthermore, we show that the PPIase pocket/domain of nuclear FKBP regulates silencing at the rDNA locus and that nuclear FKBP associates chromatin in vivo. This is the first report showing that a nuclear PPIase influences chromatin organization both in vitro and in vivo.

2.2 Experimental procedure

2.2.1 Plasmid construction and purification

Plasmids were constructed as described8. Briefly, the coding regions of SpFkbp39p and its mutant derivatives were subcloned into 6His-pET11d at the NdeI and BamHI sites51, and the coding regions of Fpr4 and mutants derivatives were subcloned into pET-28a(+) at the BamHI and XhoI sites (Novagen). Point mutations were introduced into Fpr4 plasmids by using a PCR method. Recombinant SpFkbp39p and Fpr4 proteins were expressed in E. coli with an amino-terminal histidine-tag to facilitate purification by Ni2+ affinity chromatography51. pET-6His-SpFkbp39p, pET-28a(+)-Fpr4 and constructs encoding truncated derivatives were transformed into E. coli BL21(DE3). Cells were grown to an OD600 of 0.8 at 27°C, induced with 0.4 mM isopropyl D-thiogalactopyranoside, harvested and disrupted by sonication (Branson) in buffer containing 20 mM TrisCl (pH 7.9, 4°C), 500 mM NaCl, 10% glycerol and 10 mM mercaptoethanol. The cleared lysate was applied onto ProBond resin (Invitrogen) and proteins were eluted with buffer containing 200 mM imidazole. WT, ΔC1, ΔC2 and ΔN SpFkbp39p proteins were further purified by POROS 20S ion-exchange chromatography using the BIO-CAD system (PerSeptive), POROS 20HS chromatography and dye-column chromatography, respectively. Fpr4 WT and point-mutated, ΔC, and ΔN proteins were further purified by POROS SP, histone agarose and POROS PI chromatography, respectively. The purity of the SpFkbp39p and Fpr4 WT

and truncated and point-mutated proteins was over 90 %, as estimated by SDS polyacrylamide gel electrophoresis and Coomassie staining (Figs. 1b, 3e, 4b and 7c). Recombinant FKBP12 protein was purchased from Sigma Co., Ltd. Protein concentrations were determined using the Bradford assay (Bio-Rad) using BSA as the standard.

2.2.2 Histone chaperone assay

Closed circular plasmid DNA (pBluescript) was relaxed using wheat germ topoisomerase I (Promega). The relaxed circular DNA (0.1 pmol; treated with 5 units of topoisomerase I) was combined with purified SpFkbp39p protein or other proteins (7 pmol) and HeLa core histones (52) (4 pmol) in assembly buffer (10 mM TrisCl pH 7.5, 150 mM NaCl, 2 mM MgCl2, 0.5 mM DTT and 0.1 mg/ml BSA; 50 ml final volume). Reaction mixtures were incubated at 30 °C for 1 hr, then incubated for 30 min to stop the reaction after adding an equal volume of stop A buffer (20 mM EDTA pH 8.0, 1 % (w/v) SDS and 200 mg/ml proteinase K). Plasmid DNA was extracted using phenol-chloroform, and precipitated with ethanol. The purified plasmid was then subjected to 1 % agarose gel electrophoresis.

2.2.3 Nucleosome arrays analysis

Relaxed circular DNA (0.3 pmol; relaxed with 15 units of topoisomerase I) was combined with purified recombinant SpFkbp39p (16 pmol) or other factors and HeLa core histones (20 pmol) in the assembly buffer (50 ml) described above, and then incubated at 27°C for 1 hr. To this was added 100 mM CaCl2 and MNase (1.3, 2.5 or 5 U) followed by incubation at 37°C for 1 min. The reaction was stopped by adding an equal volume of stop B buffer (20 mM EDTA pH 8.0 and 1 % SDS). Digested DNA was purified, subjected to 1.5 % (w/v) agarose gel electrophoresis and visualized by ethidium bromide staining.

2.2.4 Yeast strain and fpr4 null mutant

The genetic backgrounds of the yeast strains used are summarized in Supplementary Table 1 online. The FPR4 gene was replaced with the Candida glabrata LEU2 gene in haploid yeast (JS237) by a PCR-based procedure (53). The sequences of oligo DNA used for disruption are described in Supplementary Table 2. The fpr4::LEU2 replacement was confirmed by PCR (see Supplementary Table 2) and restriction (NdeI) analysis.

2.2.5 Immuno-fluorescence analysis to determine cellular localization

Immuno-fluorescence analysis was performed by using the method described by Pringle *et al.*[55]. Briefly, fixed yeast cells were washed with PBS containing 0.1 % Triton X-100 and 0.1 % NP-40 before antibody treatment. A 1:200 diluted anti-HA mouse monoclonal antibody (12CA5) and a 1:1000 diluted anti-Sir2p goat polyclonal antibody (Santa Cruz) was used for yeast cells (JS237) expressing HA-Fpr4p or HA-Fpr4ΔC through the *FPR4* promoter on the single copy plasmid (pRS316 derivatives). Thereafter, 1:200 diluted anti-mouse TRITC-conjugated and 1:200 diluted anti-goat FITC-conjugated antibodies were used as the second antibodies. A Laser Scanning Microscope (LSM 5 PASCAL and LSM 510, Carl Zeiss) was used to observe the immuno-stained yeast cells (57). DNA was stained with Hoechst 33342 and was visualized by a blue diode laser (Carl Zeiss).

2.2.6 Chromatin immunoprecipitation (ChIP)

The procedure for the ChIP assay has been described before (37). For the IP, Fpr4p was tagged with the Flag-epitope at its C-terminus in the native chromosome and 10 micro L of anti-Flag antibody agarose (Sigma) was used to precipitate the Flag-tagged protein from 200 mL of whole-cell extracts. Quantitative PCR was performed by using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems). SYBR Green I dye was used as a DNA binding dye for real-time detection during PCR. We used 12/1000

of the immunoprecipitates as templates in a 30 mL PCR reaction containing 50 nM of a specific primer set. To quantify the input DNA of each sample, the DNA from 0.24 ml of the whole cell extract was used. The input DNA corresponding to 0.9, 0.23, and 0.056 ml of whole-cell extracts from wild-type cells was used to construct a standard curve that would allow us to estimate the initial amount of DNA in each sample. Primer Express software (Applied Biosystems) was used to design primers that would allow various chromosomal regions to be detected. Preparation of the reaction mixtures and the PCR amplification procedure were performed according to the manufacturer's instructions.

2.2.7 Indirect end-labeling assay of chromatin at the rDNA locus

Indirect end-labeling analysis at the rDNA locus was performed as described[38]. The null mutant of the nuclear FKBP gene constructed in this study was used for the analysis. Yeast nuclei were prepared from 1 liter of SC cultures using the percoll gradient method and suspended in SPC buffer (20 mM Pipes pH 6.5, 0.1 mM $CaCl_2$, 1 M sorbitol, 1 mM PMSF). 200 µl of yeast nuclei were digested with 0, 1, 3 or 10 U of MNase at 30°C for 10 min. The DNA was then extracted with proteinase K and phenol/chloroform, digested with *Eco*R I at 37 °C O/N to determine the specific sites at the rDNA locus (38), placed at 4°C O/N and separated on an agarose gel (1.5%). The DNA fragments were then transferred to charged nylon membrane O/N (Gene Screen+; NEN) using the

aspiration blot method and cross-linked to the membrane by UV light. Southern blot hybridization using the phosphatase-labeled (Amersham) DNA probes P2 and P6 of the NTS at the rDNA locus was performed at 55°C O/N with hybridization buffer (Amersham hybridization buffer). Nonspecific binding of probes was eliminated by washing with primary washing buffer at 55 °C and secondary washing buffer as per the manufacturer's protocol. Autoradiography was performed for three minutes using a Gene Image CDP-Star detection system (Amersham). Hybridization specificity was confirmed by the lack of a signal against EcoT14I-digested λ phage DNA.

2.2.8 rDNA silencing assay

Silencing at the rDNA locus was assayed in wild type cells (JS237) or in the fpr4::LEU2 disruptant expressing full length, truncated or point-mutated versions of Fpr4p. Fpr4p expression plasmids were constructed by amplifying the FPR4 gene and 500 bp each of the promoter and terminator regions from S. cerevisiae genomic DNA and subcloning these into the single copy plasmid pRS316. Deletions and point mutations were introduced into pRS316-Fpr4, and the resulting plasmids were transformed into Δfrp4 cells. Overnight cultures of yeast strains at 30 l were harvested and RNA was extracted with the glass beads/phenol-chloroform method. RNA transcribed from MET15 integrated at the rDNA locus and from the control ADH1 gene was analyzed by RT-PCR.

2.2.9 Real time quantitative RT-PCR analysis

Quantitative one-step RT-PCR was performed using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems) and SYBR Green RT-PCR reagents (Applied Biosystems). We used 0.5 mg of the total RNA as the template in a 50 mL PCR reaction containing 50 nM of a specific primer set designed for quantitative PCR. In addition, 0.5, 0.1, 0.02 and 0.004 mg of total RNA prepared from Δfpr4 cells were used to construct a standard curve to estimate the initial amount of RNA in each sample. The target mRNA level were calculated as (reverse transcriptase (+) - reverse transcriptase (-)). The preparation of the reaction mixtures and the PCR amplification procedure followed the manufacturer's instructions. Primer Express software (Applied Biosystems) was used to design primers for detecting the genes.

2.3 Results

2.3.1 Nuclear FKBP is a histone chaperone

To determine whether nuclear FKBP directly alters chromatin structure, we examined the histone chaperone activity of SpFkbp39p8 using DNA supercoiling (24,25) and micrococcal nuclease (MNase) digestion assays (24,25) as shown in Figure 8. In comparison to buffer alone, which had no effect, SpFkbp39p induced the supercoiling of plasmid DNA in a histone-dependent manner as efficiently as the known histone chaperone NAP-I (Fig. 8a-c), which indicates that SpFkbp39p promotes nucleosome assembly. A mixture of SpFkbp39p and core histones in a 1:2 ratio was sufficient for nucleosome assembly (Fig. 8d, lane 5) and the reaction reached saturation when the molar ratio of SpFkbp39p to core histones was approximately 2:1 (Fig. 8d, lane 7). This molar ratio can be explained by the fact that core histones consist of two (H3/H4 and H2A/H2B) dimers. Therefore, SpFkbp39p reacts with core histones in a stoichiometrically relevant manner in the histone chaperone assay, which is consistent with the known properties of other histone chaperones (3).

We also examined the distribution of the nucleosomal arrays assembled by SpFkbp39p by using the MNase digestion assay. After partial MNase digestion, the DNA was extracted and analyzed by agarose gel electrophoresis. We detected mono- and di-nucleosomes (approximately 140 and 280 bp DNA fragments) in reactions containing SpFkbp39p (Fig. 8e); mononucleosomes have previously been shown to

contain approximately 146 bp of DNA (24,25). These findings suggest that SpFkbp39p assembles nucleosomes. Thus, the results of the supercoiling and MNase digestion assays demonstrate that SpFkbp39p has histone chaperone activity in vitro.

2.3.2 Novel feature as histone chaperone

During the purification of SpFkbp39p, we observed that it binds to anionic resins, which indicates that at least part of the surface of the protein is basic. This is in contrast to classical histone chaperones like NAP-I, which are acidic and thereby interact with histones. We therefore investigated whether SpFkbp39p and acidic histone chaperones differ functionally with respect to the nucleosome assembly reaction. Incubation of acidic histone chaperones with histones prior to nucleosome assembly is known to increase their activities (24,25). Thus, we determined whether this pre-incubation affects the nucleosome assembly activity of SpFkbp39p (Fig. 9a). NAP-I required pre-incubation with histones for stronger histone chaperone activity (Fig. 9b, lanes 3, 6, 9 and 12). Unexpectedly, however, SpFkbp39p did not show better histone chaperone activity upon pre-incubation. Indeed, it exhibited decreased activity after pre-incubation (Fig. 9b, lanes 2, 5, 8 and 11). This observation suggests that SpFkbp39p mediates nucleosome assembly by a mechanism that is different from that used by previously characterized histone chaperones.

2.3.3 Histone chaperone and PPIase domains

We next performed two experiments to determine whether the PPIase activity of SpFkbp39p contributes to its novel histone chaperone activity. First, we determined whether cytosolic FKBP12, which does not possess any other domains apart from the PPIase domain, exhibits histone chaperone activity. Second, we tested whether the PPIase inhibitor FK506 (26,27) also inhibits nucleosome assembly. We observed that FKBP12 did not possess histone chaperone activity (Fig. 10a,b) and that FK506 did not inhibit histone chaperone activity (Fig. 10c) at a concentration that inhibited the PPIase activity of SpFkbp39p8. Therefore, we conclude that the PPIase activity of SpFkbp39p is not essential for its histone chaperone activity.

To determine whether the N-terminal domain (1-256 residues) of SpFkbp39p is sufficient for the histone chaperone activity of the protein, we performed assays with truncated variants of SpFkbp39p (Fig. 10d,e). Both the wild type (WT) protein and an N-terminal derivative of SpFkbp39p lacking the PPIase domain (ΔC1) had histone chaperone activity, while variants consisting only of the C-terminal PPIase domain (ΔN) or a short N-terminal domain (ΔC2: 1-123 residues) did not (Fig. 10f). These data indicate that the PPIase domain is not required for the histone chaperone activity of SpFkbp30p and that the N-terminal domain is sufficient for this activity. These observations are wholly consistent with the effect of the PPIase inhibitor (Fig. 10c). Thus, nuclear FKBP is a dual-function protein that bears histone chaperone activity and

PPIase activity. Taken together, we conclude that nuclear FKBP is a novel histone chaperone in vitro.

Other histone chaperones that have been analyzed (for example, nucleoplasmin (20), N1/N2 (28), NAP-I (26), TAF-I (29), CAF-I (30) and ASF1/CIA (31-34)) have not been found to contain enzymatic domains. Thus, nuclear FKBP is the first histone chaperone found that possesses a distinct enzymatic domain. Although a PPIase inhibitor does not affect the in vitro nucleosome assembly mediated by nuclear FKBP (Fig. 10c), we did find evidence (described below) that indicates that the PPIase domain functions in vivo in chromatin organization.

2.3.4 Histone chaperone activity is conserved

Next, we investigated the role that nuclear FKBP plays in chromatin organization in vivo. To this end, we took advantage of the well-established genetic system of S. cerevisiae. SpFkbp39p and its budding yeast counterpart, Fpr435, have highly homologous primary structures (Fig. 11a). We first assessed the in vitro histone chaperone activities of Fpr4p and observed that it has the same activity as the S. pombe ortholog (Fig. 11b, c). The N-terminal domain of Fpr4p is necessary and sufficient for histone chaperone activity (Fig. 11c), which indicates that functional domain localization and, more importantly, histone chaperone activities are evolutionarily conserved among the nuclear FKBPs. The wild type (WT) Fpr4p has weaker histone

chaperone activity than the N-terminal domain alone, which indicates that C-terminal regions of the full-length protein may exert an inhibitory effect. However, the PPIase inhibitor FK506 does not affect its in vitro histone chaperone activity (Fig. 11d), which indicates the conserved domain-independency of SpFkbp39p and Fpr4p.

2.3.5 Enrichment of nuclear FKBP at the nucleolus

We reported previously that SpFkbp39p is localized in the nucleus[8]. To analyze the *in vivo* function of Fpr4p, we first had to determine its cellular localizaton. Thus, we performed an immunofluorescent assay using laser scanning microscopy, which revealed that Fpr4p localizes in the nucleus (Fig. 12a). This demonstrates that the cellular localization of this protein is conserved between *S. pombe* and *S. cerevisiae*. Interestingly, we found that Fpr4p is enriched in the nuclear subdomain (Fig. 12a), which is where Sir2p also localizes (shown as arrows in Fig. 5a, yellow spots in the merged image indicate co-localization of the two proteins). Since Sir2p has been reported to localize predominantly at the nucleolus in interphase (36), we speculated that Fpr4p also mainly localizes at the nucleolus. Confirming the nucleolar localization of Fpr4p is that we found that DNA was extensively reduced at the co-localization area (indicated as arrows in Fig .12a, DNA) because abundant rRNA is known to displace DNA in the nucleolus. Therefore, we conclude that Fpr4p is localized in the nucleus and is enriched in the nucleolus.

To investigate the *in vivo* role that the histone chaperone domain (Fpr4ΔC) of nuclear FKBP plays, it is necessary to make sure that it localizes as well as the full length Fpr4p. Thus, we next tested the localization of the Fpr4ΔC protein. The data showed that the histone chaperone domain protein of Fpr4p also localizes at the nucleus and is enriched at the nucleolus (Fig. 12b). This indicates that the histone chaperone domain is sufficient for the appropriate localization of the protein. From these observations, we predicted that Fpr4p may act at the rDNA loci that form the nucleolus, and thus we next investigated whether Fpr4p associates with the chromatin and is enriched at the rDNA loci.

2.3.6 Association of FKBP with rDNA chromatin

The nucleolus contains 100 - 200 repeats of rDNA genes that form the condensed heterochromatin. To assess the association of Fpr4p with chromatin at rDNA loci in vivo, we performed the chromatin immunoprecipitation (ChIP) assay (36). First we constructed a strain that expresses a Flag-tagged Fpr4p protein from its native chromosomal locus (Fig. 13a) and then used an anti-Flag antibody to immunoprecipitate the Fpr4p-associated DNA. In this strain, the Flag-tagged Fpr4p protein is specifically recognized by the anti-Flag antibody (Fig. 13b). We observed Flag-tag-dependent precipitation of genes at the rDNA loci, which indicates that Fpr4p associates with chromatin at the rDNA loci (Fig. 13c). In contrast, the anti-Flag antibody precipitated

the DNA at telomeric regions and the ACT1 locus two to four times less efficiently (Fig. 13c). This indicates that Fpr4p is enriched at the rDNA loci. This is the first time that it has been shown that a PPIase associates with specific chromatin areas in vivo.

2.3.7 Nuclear FKBP is required for silencing

Fpr4p associates with chromatin at the rDNA loci, which are subjected to partial gene silencing that involves the assembly of heterochromatin. Thus, we reasoned that Fpr4p may participate in the silencing of the rDNA locus. To address this possibility, we generated an fpr4 null mutation in the strain JS237, in which the reporter gene MET15 integrated at the rDNA locus is partially silenced (37). Changes in the silencing status of the rDNA locus can be estimated by measuring the level of MET15 transcription by reverse transcription (RT)-PCR analysis. MET15 expression was dramatically increased in the Δfpr4 cells compared to wild type cells (Fig. 14a, b), while ADH1 (negative control) was expressed at similar levels in both cell types. This shows that deletion of FPR4 induces a loss of silencing and indicates that endogenous nuclear FKBP is required for silencing at the rDNA locus. This provides the first evidence for an in vivo functional role for endogenous nuclear FKBP, as no phenotype had been previously found for FKBP null mutants (38).

2.3.8 Histone chaperone domain is sufficient

Since the N-terminal domain of nuclear FKBP has in vitro histone chaperone activity, we next investigated its involvement in rDNA silencing in vivo. We monitored the rDNA silencing activity in Δfpr4 cells that expressed truncated versions of Fpr4p from the FPR4 promoter on a single copy plasmid (Fig. 14c, d). Expression of the full length Fpr4p (WT) reduced the expression of MET15 integrated at the rDNA locus compared to vector alone (-) (Fig. 14c, d), which indicates that it complements the loss of silencing in Δfpr4 cells. Expression of the Fpr4p histone chaperone domain (ΔC) alone also reduced the expression of the MET15 gene at the rDNA loci (Fig. 14c, d). However, these effects were not observed in cells expressing the PPIase domain (ΔN) (Fig. 14c, d). Quantitative real time PCR analysis further demonstrated the statistically significant rDNA silencing mediated by the Fpr4p histone chaperone domain (Table 1). Thus, expression of the histone chaperone domain can suppress the loss of rDNA silencing in the fpr4 deletion. The histone chaperone domain of nuclear FKBP localizes at the nucleus and is enriched at the nucleolus. These results support a model in which Fpr4p maintains silencing at the rDNA locus through its N-terminal histone chaperone domain. Notably, however, the N-terminal histone chaperone domain silences less than the full length Fpr4p (Table 1), which suggests that the C-terminal PPIase domain plays some role in rDNA silencing in vivo.

2.3.9 Nuclear FKBPs modulate chromatin structure *in vivo*

Since gene silencing is affected by chromatin structure, we next investigated whether endogenous nuclear FKBPs participate in altering chromatin structure at the rDNA locus by using the indirect end-labeling assay. Here, yeast nuclei were isolated and treated with increasing amounts of micrococcal nuclease (MNase) to digest linker DNA between nucleosomes, and the extracted DNA was analyzed by Southern blot hybridization using a rDNA probe comprised of non-transcribed spacer (NTS) sequences from the DNA locus (40). If the conformation of chromatin is changed at specific sites, we expect to observe alterations in the MNase-sensitivity of the NTS sites.

The MNase cleavage pattern of Δ*fpr4* chromatin differed from that of the WT at the rDNA locus (Fig. 15a). As seen in lanes 1-4 relative to lanes 5-8, the NTS1 sites indicated by closed arrows were cleaved by MNase more efficiently in Δ*fpr4* chromatin than in WT chromatin (Fig. 15a). The sites indicated by open arrows in Δ*fpr4* chromatin were cleaved less efficiently (Fig. 15a). These differences were not observed in the NTS2 region (Fig. 15b) and the amounts of total DNA were same (Fig. 15c), which indicates the specific alteration of MNase-sensitivity. These data indicate that the rDNA chromatin structure in Δ*fpr4* cells differs from that in WT cells. This result represents the first demonstration that endogenous PPIase affects chromatin structure *in vivo*. This notion is consistent with the finding that Δ*fpr4* null mutations affect silencing at the rDNA locus. Thus, endogenous nuclear FKBPs appear to participate in regulating silencing by associating with chromatin and altering the chromatin structure at the rDNA locus *in vivo*.

The data described above indicate that while Fpr4p associates with chromatin at both NTS1 and NTS2 regions as shown by the ChIP experiment (Fig. 13), *FPR4*-dependent alteration of chromatin structure is observed only at the NTS1 region (Fig. 15). The NTS1 region is known to include enhancer activity, and *SIR2*-dependent alteration of chromatin structure has also been reported at the NTS1 region but not the NTS2 region (40). Fpr4p exists at the NTS2 region but its disruption does not alter chromatin structure there. It may be that other molecule(s) participate in altering the chromatin structure in the NTS2 region.

2.3.10 The in vivo role played by PPIase domain

In addition to the demonstrated requirement for the N-terminal histone chaperone domain, we postulate that the C-terminal PPIase domain/activity also plays a non-essential regulatory role in rDNA silencing in vivo. To investigate this, we generated point mutations of evolutionarily conserved amino acids in the PPIase domain that have been reported to affect substrate binding and PPIase activity (39) (summarized in Fig. 16a, Table 2). The substitution of phenylalanine 323 with tyrosine (F323Y) has been reported to have the strongest effect on PPIase activity (an approximately four-fold reduction), while substitution of aspartic acid 324 with valine (D324V) did not affect PPIase activity in a protein substrate assay (39) (Table 2).

We found that the FPR4 F323Y mutation stimulated rDNA silencing while the

D324V mutation did not (Fig. 16b). Furthermore, the substitution of phenylalanine 323 with alanine (F323A) also stimulated silencing, which confirms the importance of the FPR4 phenylalanine 323 side chain in rDNA silencing (Fig. 16b). Quantitative analysis using another set of primers designed for real time PCR demonstrated the statistically significant enhancing effect of the Phe323 mutations (Table 2). These results suggest that nuclear FKBP may regulate rDNA silencing through the PPIase activity or pocket. Since all the purified point mutated nuclear FKBP proteins (Fig. 16c) possess in vitro histone chaperone activities that are as good as wild type activities (Fig. 16d), we speculate that endogenous proteins and/or ligands other than histones may interact with the PPIase pocket/domain of nuclear FKBP and thereby mediate signals that are relevant to silencing. Taken together, these findings indicate that nuclear FKBP consists of two separate domains: the histone chaperone domain that is essential for rDNA silencing and the PPIase domain that regulates the latter property (Fig. 17). The existence of these two functionally distinct domains is a novel property for a chromatin factor.

2.4 Discussion

2.4.1 Significance of nuclear FKBP

PPIases have not previously been considered to participate in altering nucleosome structure. We show here that 1) nuclear FKBP has in vitro histone chaperone activity at stoichiometrically relevant concentrations, 2) the histone chaperone activity of nuclear FKBP depends on its N-terminal domain and is independent of PPIase activity of the C-terminal domain, 3) nuclear FKBP associates with chromatin at rDNA loci and plays an in vivo functional role in rDNA silencing, as demonstrated by ChIP, mutation and suppression analyses, and 4) the C-terminal PPIase domain of nuclear FKBP is not essential for the silencing and histone chaperone activity of the protein but it regulates rDNA silencing in vivo. These results provide the first evidence that endogenous nuclear FKBP participates in rDNA silencing through the histone chaperone domain that we identified (Fig. 17).

We found significant differences in the primary structures of nuclear FKBP and classical histone chaperones. The histone chaperone domain of nuclear FKBP has a basic region that is not present in other histone chaperones (Fig. 8) and which is assumed to interact with acidic molecules such as DNA and the acidic domains of nuclear proteins. Furthermore, we have shown that there are differences in the nucleosome assembly reactions mediated by nuclear FKBP and the classical histone chaperone NAP-I (Fig. 9). Taken together, we postulate that the nucleosome assembly/disassembly reactions carried out by PPIase differ mechanistically from those

of other classical histone chaperones.

The nucleosome assembly/disassembly reactions mediated by histone chaperones can be broken down into several steps: association and dissociation of histones H3/H4 with and from DNA, association and dissociation of histones H2A/H2B with and from histones H3/H4, association and removal of histones with and from DNA, and so on (2). Thus, the functional differences between PPIase and classical histone chaperones can be resolved by identifying the step(s) of nucleosome assembly/disassembly reactions that they affect and the other chromatin factors with which they cooperate at each step.

2.4.2 Nuclear FKBP in silencing

In addition to the nuclear FKBP reported here, other histone chaperones, including ASF1/CIA and CAF-I, also contribute to silencing at the rDNA locus (30,41). The way in which distinct histone chaperones relate to each other must be understood in order to determine why different types of histone chaperones function in silencing at the rDNA locus, as the cooperative activity between different classical histone chaperones may be important. For example, although ASF1/CIA and CAF-I bind to different regions of the histone cores, they have similar histone chaperone activities and work cooperatively in DNA replication reactions in vitro (31). Clearly, the putative functional interactions between classical histone chaperones and PPIases warrants additional investigation.

Fpr4 is required for the silencing of a reporter gene integrated at the rDNA locus

(Fig. 15), and the point mutations that impair the PPIase activity of Fpr4 appear to enhance the silencing of the reporter gene at the rDNA locus in vivo (Fig. 16 and Table 2). The null mutant of the nuclear FKBP is a loss of function mutant and these point mutants should be considered as gain of function mutants. Such cases are well known in the genetics in various organisms (for example, p53 in mammals (42)). These results suggest that a putative endogenous factor interacts with the C-terminal PPIase domain. We showed here that the Δfpr4 null mutant affects rDNA silencing; this phenotype is known to be induced by mutations in several chromatin factors such as the silencing factor Sir4 and the NAD-dependent histone deacetylase Sir2 (36). Furthermore, we confirmed that Δfpr4 has altered sensitivity to MNase digestion in the NTS at the rDNA locus (data not shown). Similarly, the Δsir2 mutant also has increased sensitivity to MNase in the NTS (43). Therefore, nuclear FKBPs could interact with the other chromatin factors physically and/or functionally (Fig. 17). While nuclear FKBP associates with chromatin at both the NTS1 and NTS2 regions (Fig. 13), the alteration of chromatin is dependent on Fpr4 only with regard to the NTS1 region (Fig. 15). This suggests that another additional positive chromatin factor(s) is necessary for the alteration of chromatin structure at the NTS2 region or that a negative chromatin factor(s) must be suppressed before Fpr4 can function in this region. To understand the mechanism by which the rDNA locus is silenced, the functional relationships between the PPIases and the other chromatin factors must be investigated. For this, it will be necessary to identify the chromatin factors that interact with nuclear FKBP.

2.4.3 PPIase domains and other domains

We have shown here that nuclear FKBPs have dual functions consisting of histone chaperone and PPIase activities. Other nuclear PPIases possess different additional domains. For example, Ess1/Pin1 and FKBP52 possess WW and Tpr domains, respectively, in addition to the PPIase domain (44,45), and these domains facilitate the identification of targets of their PPIase domains (42,44-46). For example, the WW domain of Pin1 interacts with mitotic factors and RNA polymerase II, including phospho-serine/threonine proline sequences (47), and the Tpr domain of FKBP52 binds to molecular chaperones such as hsp70 and hsp9048. Although the additional domain of FKBP51 is independent of the PPIase domain in vitro, both domains are necessary for its full activity in vivo, as has been demonstrated for nuclear FKBP (11-13). These observations suggest a close relationship between PPIase domains and these additional domains with respect to their reactions. This model is supported by our observation that the C-terminal PPIase domain regulates silencing in vivo, while the N-terminal histone chaperone domain is essential for silencing (Fig. 16).

PPIase domains have been found to catalyze and/or interact with several transcription factors (7,9-19). Here, we have shown that the additional N-terminal domain has histone chaperone activity. Thus, analogous to the way in which transcriptional activators link DNA elements (which are the targets of their DNA-binding domains) to the transcriptional machinery (which is the target of their activation domains), nuclear FKBPs may bridge histone-containing chromatin complexes (which are targets of the N-terminal histone chaperone domain) and proline-containing nuclear

factors (which may be targets for the PPIase domain) (Fig. 17). We propose that cooperation between the histone chaperone domain/activity and the PPIase domain/activity could efficiently regulate chromatin structure.

2.4.4 Perspectives for nuclear PPIases

The in vitro MNase assay data (Fig. 8e) demonstrate nucleosome arrays up to only two nucleosomes. Similarly, the nucleosome spacing assembled by histone chaperones in vitro is not clearly ordered (4,25,32). Histone chaperones assemble ordered nucleosomes together with ATPase (e.g. ACF1) and present a clear nucleosomal ladder in the MNase assay in vitro4. Thus, we believe that nuclear FKBP also cooperates with ATPase in vivo. Identification of the partner of nuclear FKBP that is involved in ordering the nucleosomes would be of interest.

We postulate that nuclear PPIases also participate in aspects of chromatin organization other than rDNA silencing, because PPIase targets are present in various nuclear factors. For example, 1) several transcription and nuclear factors bear various types of proline-rich domains, 2) a nuclear PPIase, human Pin1, associates with the proline-rich carboxyl terminal domain of a hyperphosphorylated form of the largest subunit of RNA polymerase II (49,50), and 3) Ess1, a yeast homologue of Pin1, is linked to chromatin remodeling complexes and to the general transcription machinery (16,17). Considering this evidence, we believe that further analysis of nuclear FKBP

function will considerably improve our understanding of the regulation of chromatin transcription.

Conclusion

In the 1st section, I proposed a link between the localization and primary structure of FKBPs, and provided evidence for this association by isolating and localizing a novel nuclear FKBP. I also predicted, on the basis of its domain structure, that the nuclear FKBP would function towards DNA and histones.

In the 2nd section, I provide evidence for other predictions raised in the 1st section. Nuclear FKBP is a novel type of histone chaperone *in vitro* and is involved in rDNA silencing *in vivo*. Domain analyses indicated that the histone chaperone domain of nuclear FKBP is sufficient for rDNA silencing. These analyses are the first demonstration of a function for FKBPs in chromatin organization. Here, I propose a concept whereby PPIase is linked to the alteration of chromatin structure.

I was able to identify this link between FKBP and chromatin by focusing on the primary structure of FKBPs. Function cannot be separated from structure and must be related to structure. Thus, to investigate functions of unknown factors, I think it is important to study their primary structures in detail.

Perspective

1. Mechanism of histone chaperoning by nuclear FKBP

The activity of classical histone chaperone is induced by preincubation with histone. However, nuclear FKBP has activity in the absence of histone. Thus, I propose that nuclear FKBP has another target other than histone. Given that the nucleosome consists of DNA and histones, I investigated the possibility that nuclear FKBP would function towards DNA. Here, I analyze the action of nuclear FKBP on DNA.

The results obtained suggested the existence of two types of histone chaperones. By finding both commonality and variety among histone chaperones, I will further elucidate the role of histone chaperones in the alteration of chromatin structure.

2. Significance of PPIase domain

The introduction of point mutations in the PPIase domain of nuclear FKBP enhanced rDNA silencing. However, histone chaperone activity was unaffected. This suggests that the PPIase domain may act on certain chromatin proteins.

I predict the candidate targets of the PPIase domain as follows:

Histone

As a major component of chromatin, histone is a candidate target of the PPIase domain. However, since point mutation of the PPIase domain does not affect histone chaperone activity, other targets must exist. Since PPIase is known to physically interact with several chromatin factors, the PPIase domain of nuclear FKBP may target these chromatin factors.

Transcriptional machinery

I commenced this study because I had identified FKBP as a TFIID interactor. The C-terminal domain of the largest subunit of RNA polymerase II interacts with Pin1. TFIID alter its structure when transcription is activated. Thus, analyses of PPIase in transcription may uncover novel mechanisms of transcriptional activation.

DNA-binding transcriptional activators

Several transcriptional activators possess proline-rich domains, which may be targets of PPIase.

Through interactions between the PPIase domain and the factors described above, nuclear FKBP may play a role in linking chromatin to various chromatin factors. Moreover, both histone chaperone and PPIase domains can alter molecular structures, suggesting that nuclear FKBP could alter chromatin structure efficiently.

3. Alteration of DNA structure by nuclear FKBP

Alteration of DNA structure is important in regulating access to DNA of proteins that catalyze gene expression. Changes in DNA structure are effected through enzymes like topoisomerases and various DNA-binding proteins. Previously, PPIases have been found to participate in altering protein structures. Here, I found that nuclear FKBPs also participate in altering DNA structure. Although, this data is not included in this thesis, it is summarized as follows:

The charge of the surface of SpFkbp39p is basic, suggesting that this protein could bind to DNA. To test this possibility, I performed a DNA binding assay using DNA-cellulose. The results obtained indicated that SpFkbp39p and histones bound DNA, while CIB protein did not. Next, I investigated how SpFkbp39p could affect the structure of DNA. Using a supercoiling assay, I tested the change in the linking number of circular DNA induced by SpFkbp39p in the absence of histone and in the presence of topoisomerase I. The results showed that SpFkbp39p decreased the amount of relaxed DNA and increased the amount of negatively supercoiled DNA which had a linking number of one to three (Fig. 18). This suggests that one molecule of circular DNA wraps around one to three molecules of SpFkbp39p. Next, I investigated the relationship between nucleosome assembly activity and alteration of DNA structure by examining the dose-dependent effects of SpFkbp39p on these activities. Both activities increased in a dose-dependent manner and were correlated (Figure 19). Alteration of DNA structure requires lower concentrations of SpFkbp39p than for nucleosome assembly. Thus, the association of SpFkbp39p with DNA might be a trigger for nucleosome assembly. Mediation of DNA structure alteration was observed only for the full-length, but not for the N- or C-terminal portion of the protein, indicating that the PPIase domain is required for the alteration of DNA structure. It is surprising that the PPIase domain contributes to conformational changes not only in protein, but also in DNA.

When chromatin DNA is assembled, many DNA binding proteins in addition to histones are included in the chromatin DNA complex. Several DNA binding proteins including TBP (TATA box-binding protein, a general transcription factor) and HMG

(high mobility group, non-histone chromatin component) have been reported to prefer negatively supercoiled DNA[22] for their activities. SpFkbp39p might facilitate such factors by binding to chromatin DNA and altering DNA structure.

These *in vitro* observations demonstrate that nuclear FKBPs alter nucleosomal, DNA and protein structures (Fig. 20 & 21). However, I have not yet obtained *in vivo* evidence for this phenomenon. In my opinion, analyses of null mutants of nuclear FKBP genes will indicate that endogenous nuclear FKBPs also participate in the alteration of DNA structure.

4. Role of nuclear FKBP in silencing and aging

Since histone deacetylase SIR2 and RPD3 are involved in rDNA silencing, I investigated whether nuclear FKBP interacts with them genetically and/or physically.

rDNA silencing is closely related to aging. Thus, nuclear FKBP may also contribute to aging. Here, I will analyze functional interactions between nuclear FKBP and the aging factors, SIR2/3/4 and SGS1, in yeast and mouse models. Furthermore, I will analyze whether alteration of DNA structure by nuclear FKBP is related to the presence of extra chromosomal DNA, which is an aging marker (Fig. 22).

5. Nuclear FKBP and other chromatin factors

The catalytic domain of SpFkbp39p is not essential for its nucleosome assembly activity. Thus, I speculated that this catalytic domain might work cooperatively with the N-terminal region of SpFkbp39p and other chromatin factors. To investigate which factors contain acidic and basic regions like those of SpFkbp39p, I searched for factors homologous to the N-terminal region of SpFkbp39p using BLAST and PSI-BLAST programs. Surprisingly, various types of chromatin factors were found to contain acidic and basic regions similar to those of SpFkbp39p (75 chromatin factors / 238 identified factors), for example, histone deacetylase, N-CoR, the CAF1 p150 subunit, histone H1, topoisomerase, DNA methylase, chromodomain ATPase, centromere binding protein etc. Although none of these factors possess a clear consensus motif, their common activity might be mediated by the putative flexible structures present in their charged regions. In view of these considerations, SpFkbp39p might work on chromatin DNA in tandem with these chromatin factors. Thus, I predict that PPIase activity is not required in the nucleosome assembly reaction but is needed to regulate the more complex organization of chromatin.

I found preliminary evidence that the PPIase domain can work on the transcriptional machinery in yeast cells by isolating PPIase as a novel interaction factor of the general transcription factor TFIID. From these results, I propose that transcription factors and/or the transcriptional machinery is associated with, and regulated by, PPIase domains and activities, respectively.

6. Conservation of nuclear FKBPs.

Nuclear-type FKBPs exist in many species from yeast to mammals and all have N-terminal charged regions in addition to PPIase domains. Most of their sequences are not highly conserved, with the exception of the catalytic domain, but interestingly, they all contain acidic and basic regions in their N-termini, suggesting that the activities described above might be conserved among nuclear-type FKBPs. In comparison to other types of nuclear FKBPs, FKBP25, a human nuclear-type FKBP, has a basic region but not a typical acidic region in its N-terminus. However, FKBP25 is known to be phospholyrated by casein kinase II, which would increase its acidity. Thus, in mammals, nucleosome assembly activity by nuclear FKBP25 might be regulated by phosphorylation, perhaps reflecting the complexity of differentiation and development in mammals.

The *Saccharomyces cerevisiae* homologues of SpFkbp39p, Fpr3 and Fpr4, are not essential for yeast cell growth. I propose that these FKBPs may contribute to specific chromatin events because several other chromatin factors, such as GCN5 and SWI2, are also not essential for viability but are known to regulate the expression of specific genes. In higher eukaryotes, several PPIases have been reported to be required for specific developmental stages. Considering these results and the conserved features of their primary structures, I propose that the regulation of specific chromatin DNA organization by nuclear PPIases is a common mechanism for diverse species from protozoa to metazoa.

Therefore, to advance the state-of-the-art on FKBPs, I would like to propose the following experiments:

1. Identification of the targets molecules for FKBPs. I plan to identify target proteins interacting with the nuclear FKBPs using protein column and two-hybrid systems. This will indicate how nuclear FKBPs affect the conformation of their target proteins.
2. Determining the relationship between isomerization of the prolyl imide-bond and alteration of chromatin structure. I will investigate how isomerization of prolyl imide-bonds affects the alteration of chromatin structure.
3. The interactions of PPIases with transcriptional machinery. I will also investigate how PPIases affect transcriptional machinery and gene expression.
4. The substrates of FKBP, cyclophilin and Pin1 are restricted, implying the existence of other types of PPIases. Thus, I will search for new types of PPIases.
5. Finally, I will investigate the relationship between the rotation of the C alpha residue and isomerization of proline.

Epilogue

It is unclear why PPIases exist in all species and why two types of PPIases (FKBPs and cyclophilins) are not essential for growth. Through the above analyses, I intend to resolve these issues. Finally, my ultimate goal will be to elucidate the underlying basis of conformational changes of proteins in biological reactions.

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Takashi Kuzuhara

Tokyo, Japan

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Table 1: The effects of the nuclear FKBP deletion mutations on rDNA silencing.

Expressed deletion-mutants and WT proteins	rDNA silencing (*MET15* mRNA normalized to the WT level)
- (vector)	43 ± 5.1
WT	1.0 ± 0.083
ΔC	4.2 ± 2.3

Quantitative RT-PCR to measure levels of expression of the *MET15* gene for rDNA silencing. Target mRNAs from cells expressing wild type or deletion mutants Fpr4p were detected by quantitative RT-PCR of the total RNA prepared from each strain. The results were normalized with regard to the level in cells expressing the wild type (mean ± standard deviation from duplicate samples).

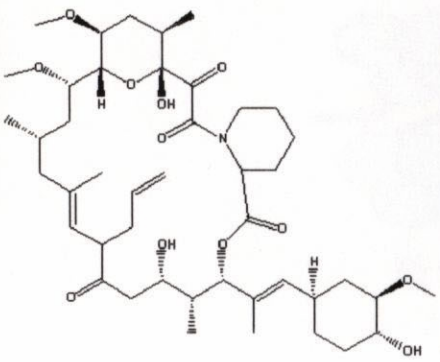
Table 2: The effects of the nuclear FKBP point mutations on rDNA silencing and PPIase activities.

Expressed point mutants and WT proteins	rDNA silencing (*MET15* mRNA normalized to the WT level)	PPIase activity (Analysis using protein substrate[39])
F323Y	0.36 ± 0.13	13,000
D324V	1.6 ± 0.1	49,000
F323A	0.14 ± 0.10	N.D.
WT	1.0 ± 0.083	51,000
- (vector)	43 ± 5.1	-

The PPIase activities of the FKBP mutants F323Y and D324V have been analyzed previously[41]. Quantitative RT-PCR to measure expression levels of the *MET15* gene for rDNA silencing. Target mRNAs from cells expressing wild type or point mutants Fpr4p were detected by quantitative RT-PCR of the total RNA prepared from each strain. The results were normalized with regard to the level in cells expressing wild type (mean ± standard deviation from duplicate samples).

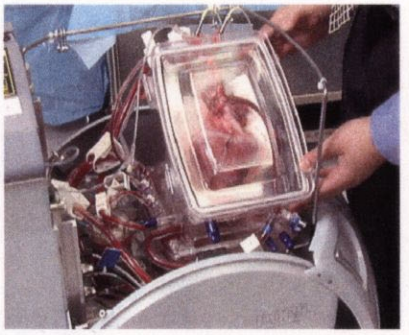
免疫抑制剤FK506

化学構造式



日本の藤沢薬品で独自に開発された
免疫細胞の活性を抑制する薬
筑波山の土壌中のバクテリアから
単離された。(1984)

適応：
1) 臓器移植における拒絶反応の抑制
2) アトピー性皮膚炎（患者数十万人）
3) 慢性間接リューマチ
4) 炎症性腸疾患
5) アレルギー性結膜炎
6) 乾癬



臓器移植時の拒否反応の抑制

FKBP: FK506結合蛋白質

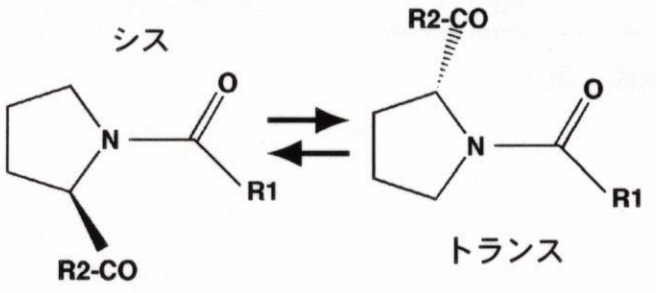
FKBP - FK506複合体

プロリン異性化酵素(PPIase)

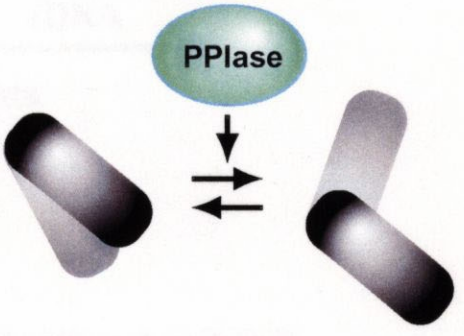
PPIase
- FKBP family
  inhibitor : FK-506
- Cyclophilin family
  inhibitor : Cyclosporin A
- parvulin family
  inhibitor : n.d.

PPIaseの酵素活性

プロリンの軸の回転

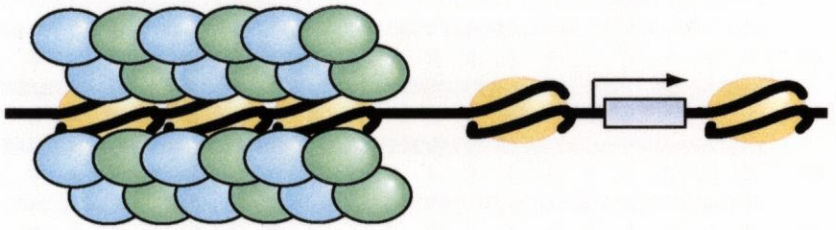


蛋白質の構造変換



Introduction figure 1 免疫抑制剤FK506とFKBP

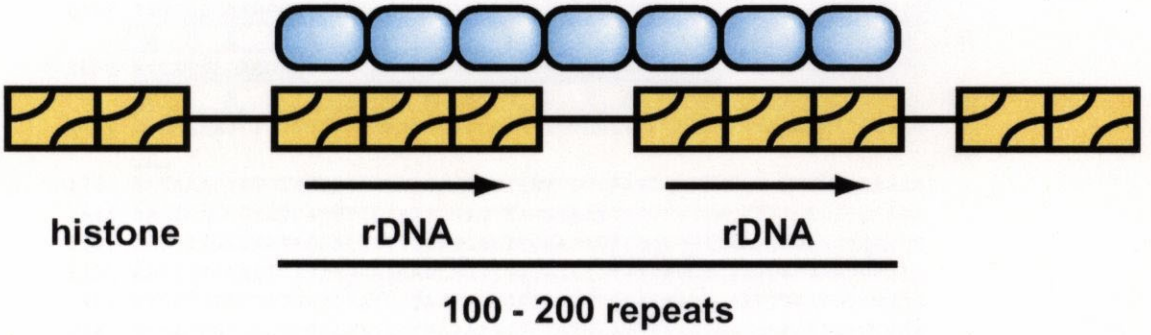
染色体からの遺伝子発現制御



眠っている遺伝子　　起きている遺伝子

染色体構造変換 (ヌクレオソーム構造変換反応)

rDNA領域: 遺伝子発現が抑制されている



Introduction figure 2 クロマチン構造変換と遺伝子発現

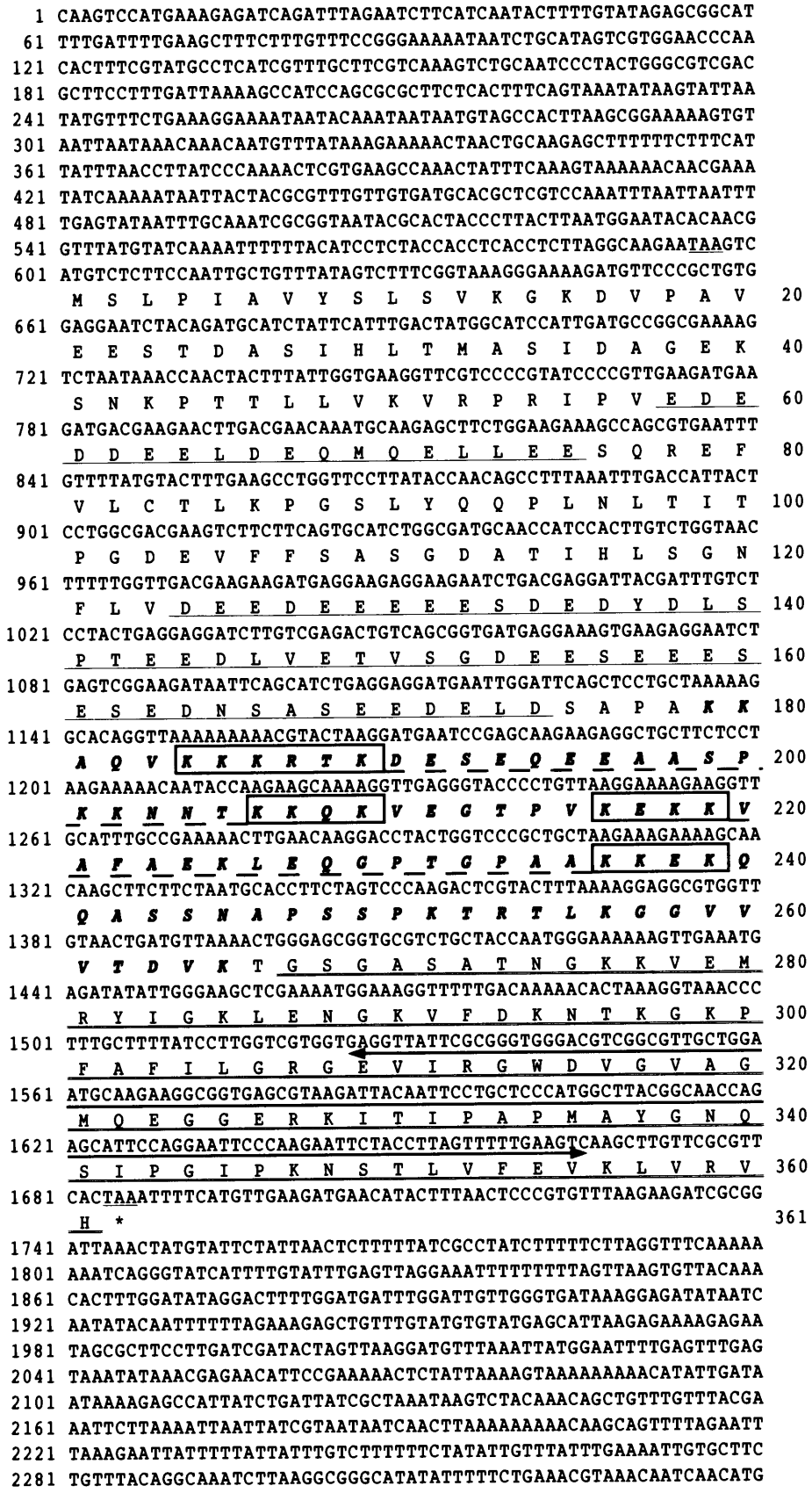


Figure 1. Nucleotide and deduced amino acid sequences of the cloned Sp*fkp39+* gene. Numbers of nucleotide sequence from the 5'-terminal are shown on the left and amino acid sequence from the N-terminal on the right. The arrow at nucleotide position 1525-1665 indicates the position of the DNA fragment we first obtained by PCR using degenerate oligonucleotide primers. In-frame nonsense codons (TAA) at nucleotide position 595-597 and 1684-1686 are underlined. The C terminal region homologous to catalytic domains of FKBPs is double underlined (at amino acid position 267-361). One basic (at amino acid position 179-265) and two acidic (positions 58-75 and 124-174) regions are shown underlined and in bold, respectively. Consensus bipartite nuclear targeting sequences within the basic region are boxed (basic stretch) and dotted-lined (linker) (at amino acid positions 184–209 and 215-239).

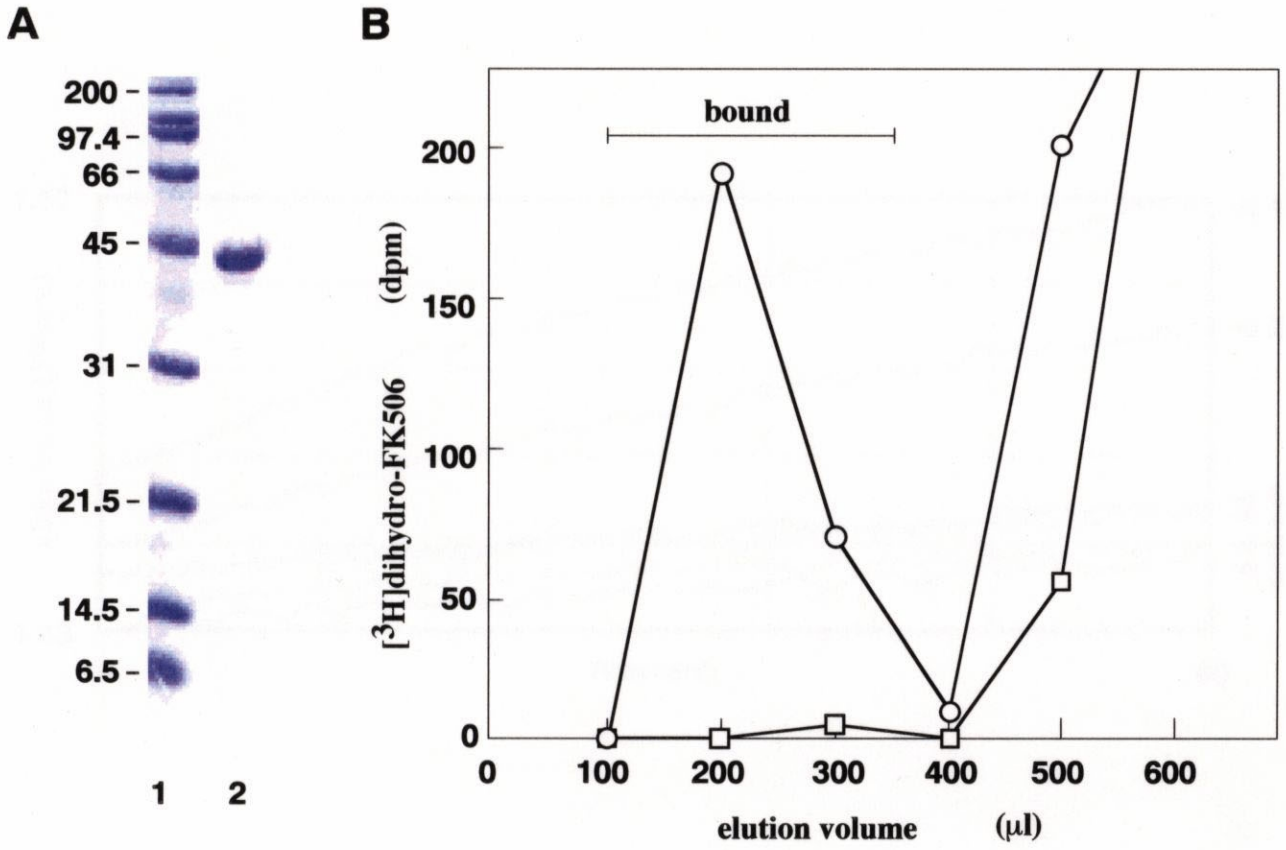


Figure 2. The interaction between SpFkbp39p and FK506. A. Purity of recombinant SpFKBP39p protein. Lane 1, protein standard (kDa); lane 2, purified SpFKBP39p. Recombinant SpFKBP39p protein (1μg) was resolved by 12.5% SDS-polyacrylamide gel. Gel was stained with Coomassie blue. B. Binding of [3H]dihydro-FK506 to SpFKBP39p. [3H]dihydro-FK506 was incubated in the presence (circle) or the absence (square) of SpFKBP39p and fractionated in LH-20 gel filtration chromatography. [3H]dihydro-FK506-bound fractions are eluted in void.

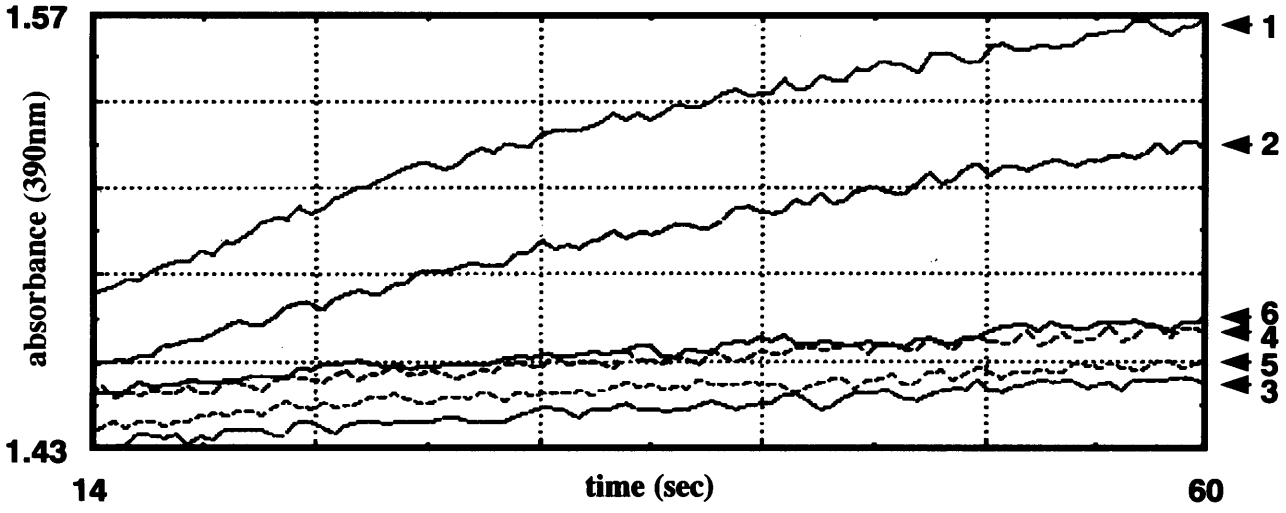


Figure 3. Peptidyl-prolyl *cis-trans* isomerase activity of SpFKBP39p and its inhibition by FK506. The absorbance at time t was plotted against time. Line 1, SpFKBP39p (52nM); line 2, HsFKBP12 (17nM); line 3, enzyme(-); line 4, SpFKBP39p + FK506 (10μM); line 5, HsFKBP12 + FK506; line 6, enzyme(-) + FK506.

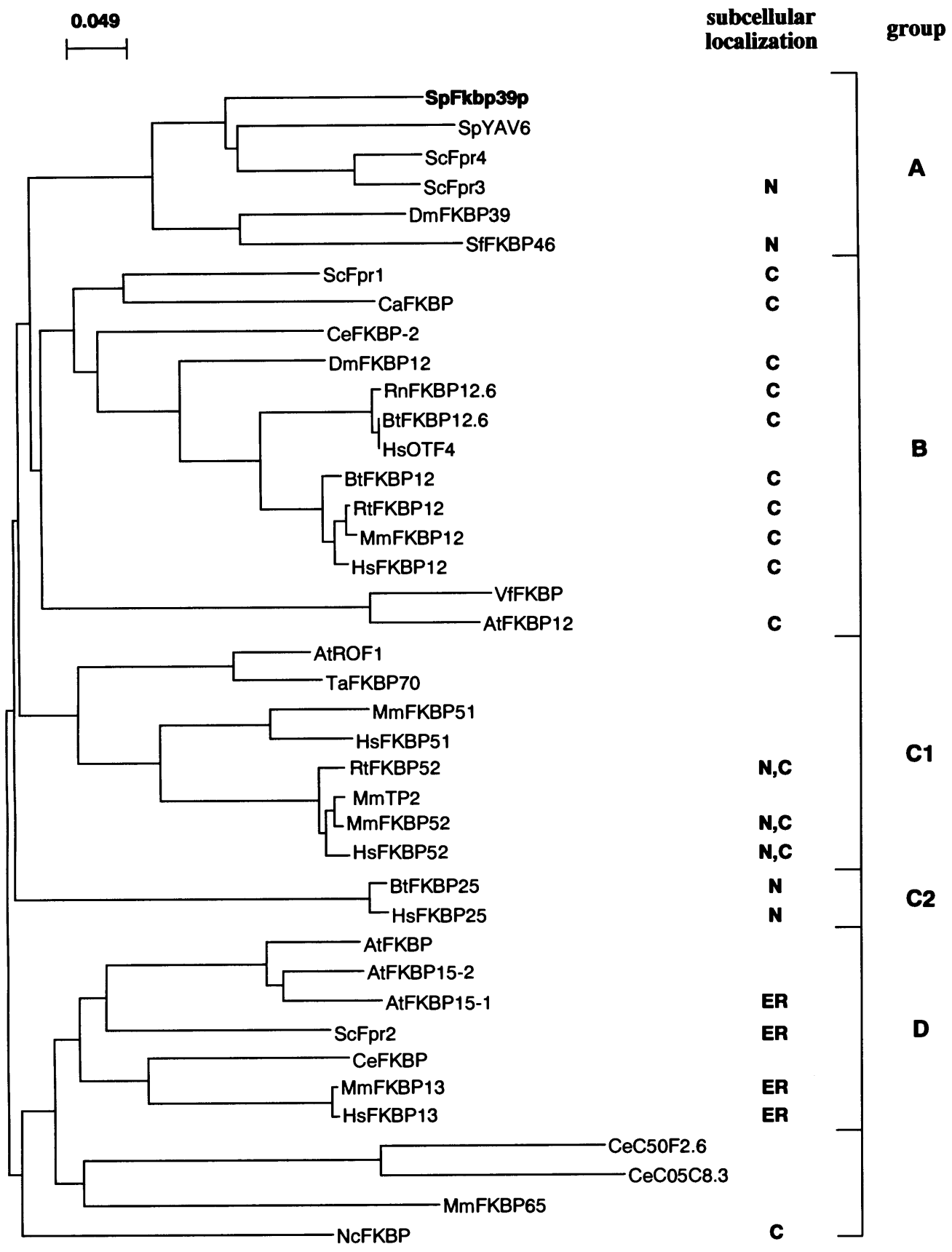


Figure 4. Phylogenetic tree analysis of PPIase catalytic domains of eukaryotic FKBPs. This tree is determined by using CLUSTAL W and NJPLOT programs (45) by primary sequences of PPIase catalytic domains. The symbols N (nucleus), C (cytoplasm) and ER (endoplasmic reticulum) represent the subcellular localization of FKBPs, which were previously determined.

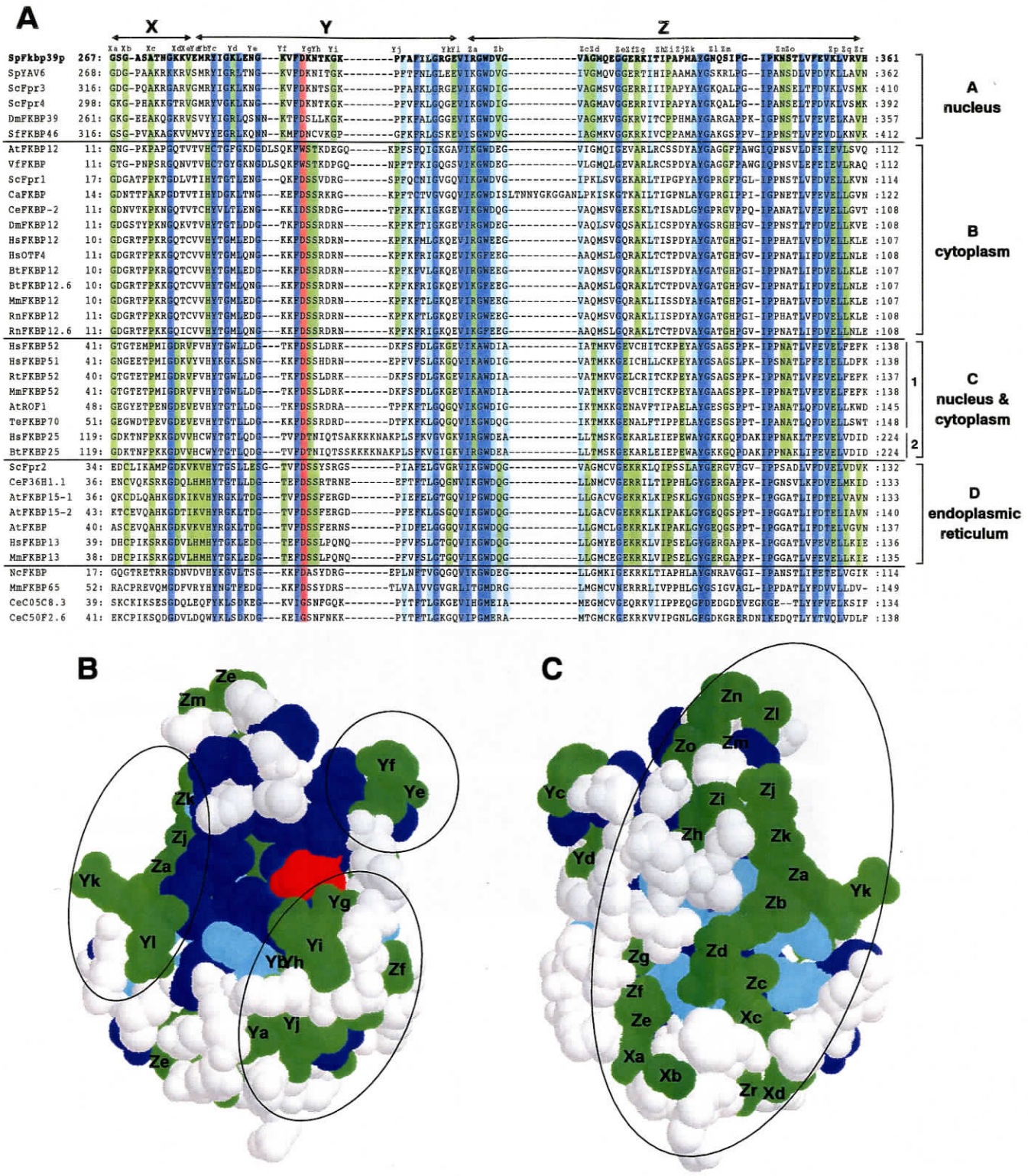


Figure 5. Detailed analysis of amino acid residues of catalytic domains. A. Alignment of amino acid sequences of the PPIase catalytic domains of the FKBPs including SpFkbp39p. The alignment was determined using CLUSTAL W program (45). "Conserved" residues are blue-shadowed (conserved over 90% identity) and cyan-shadowed (100% similarity). Residue D37 (HsFKBP12) which is essential for full PPIase activity (51) is red-shadowed. "Group-specific" residues, which are completely identical and similar within one, two or three group(s), are green-shadowed. Three regions, whose "group-specific" residues are closely located at the three-dimensional level, are named as regions X, Y and Z, respectively (indicated by arrows). Letters above the alignment show the positions of the amino acid residues in the three-dimensional structures of HsFKBP12 as discussed in B and C. B. "Conserved" and "group-specific" residues mapped on the corresponding positions of the three-dimensional structure of HsFKBP12. Amino acids shown by colors and letters correspond to those of A. "Group-specific" region and sites are indicated by circles. C. "Conserved" and "group-specific" residues viewed from another angle. Amino acids shown by colors and letters correspond to those of A and B. "Group-specific" region and sites are indicated by circle.

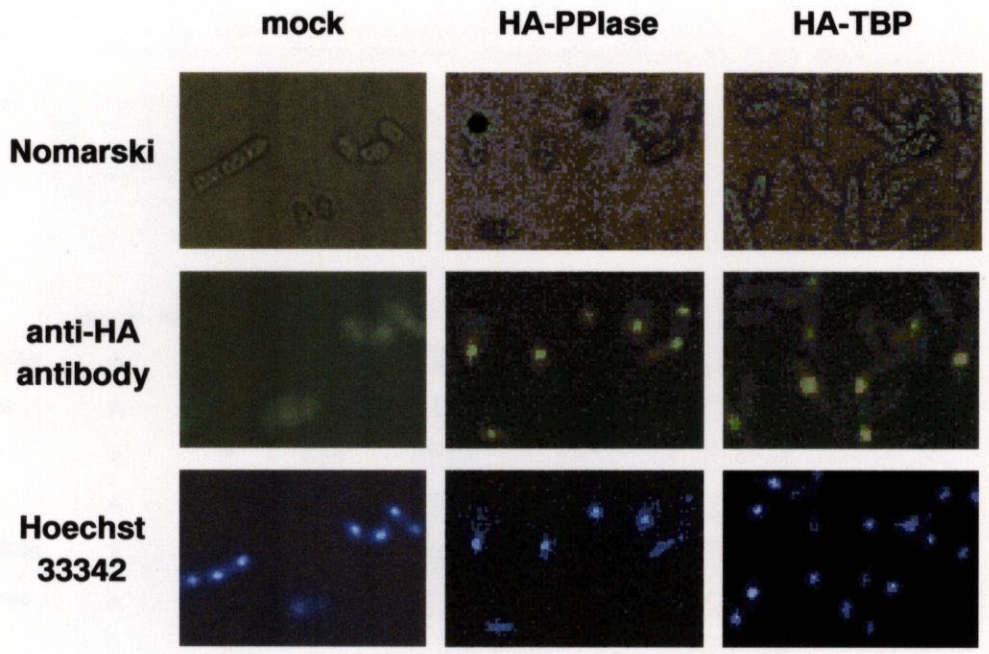


Figure 6. Nuclear localization of HA-SpFkbp39p. Strains carrying pREP1, pREP1-HA-Fkbp39p and pREP1-HA-TBP plasmids were stained with anti-HA antibody. Photographs of cells showing Nomarski (upper), anti-HA antibody immunofluorescence (middle) and Hoechst 33342 fluorescence of DNA (lower).

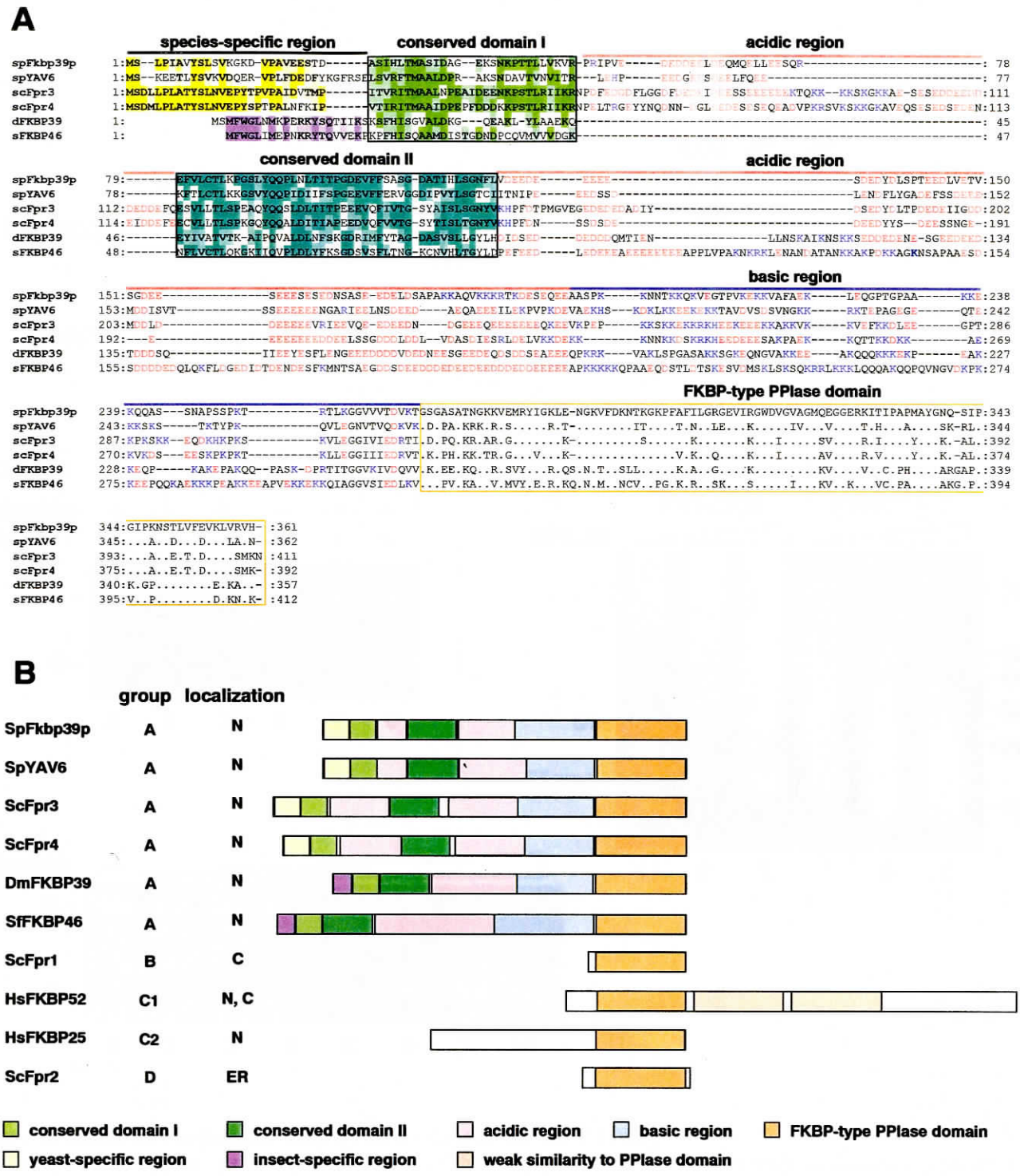


Figure 7. Characteristic domain structure of group A FKBPs. A. Alignment of the primary structures of group A FKBPs including SpFkbp39p. The alignment was carried out using CLUSTAL W program (45). Conserved domains I, II and the PPIase catalytic domain are boxed, respectively. In conserved regions I and II, identical and similar amino acids are dark- and pale-shadowed, respectively, and in the PPIase domain, the amino acids identical to those of SpFkbp39p are illustrated by dots. Acidic and basic regions are red and blue lined above, respectively. The acidic (D, E) and basic (K, R and H) residues in charged regions are red and blue. The species-specific region where the primary structures of yeast and insect (yellow and purple colored, respectively) group A FKBPs are different from each other is present in the most N-terminal region, and identical and similar amino acids within yeasts or insects are dark and pale shadowed, respectively. B. Summary of domains and regions which are present in FKBPs. Group A FKBPs have conserved domains I (indicated by pale green) and II (dark green), acidic (red) and basic (blue) regions, and the PPIase domain (orange). At the most N-terminal region, there are "species-specific" regions (indicated by yellow and purple in yeasts and insects FKBPs, respectively) whose primary structures of yeasts and insects group A FKBPs are different from each other. Note that position of each domain and region are in order. These characteristic domains are not present in groups B, C and D. HsFKBP52 (group B2) has two domains which have weak similarity to the FKBP-type PPIase catalytic domain (indicated by pale orange) in addition to the functional catalytic domain.

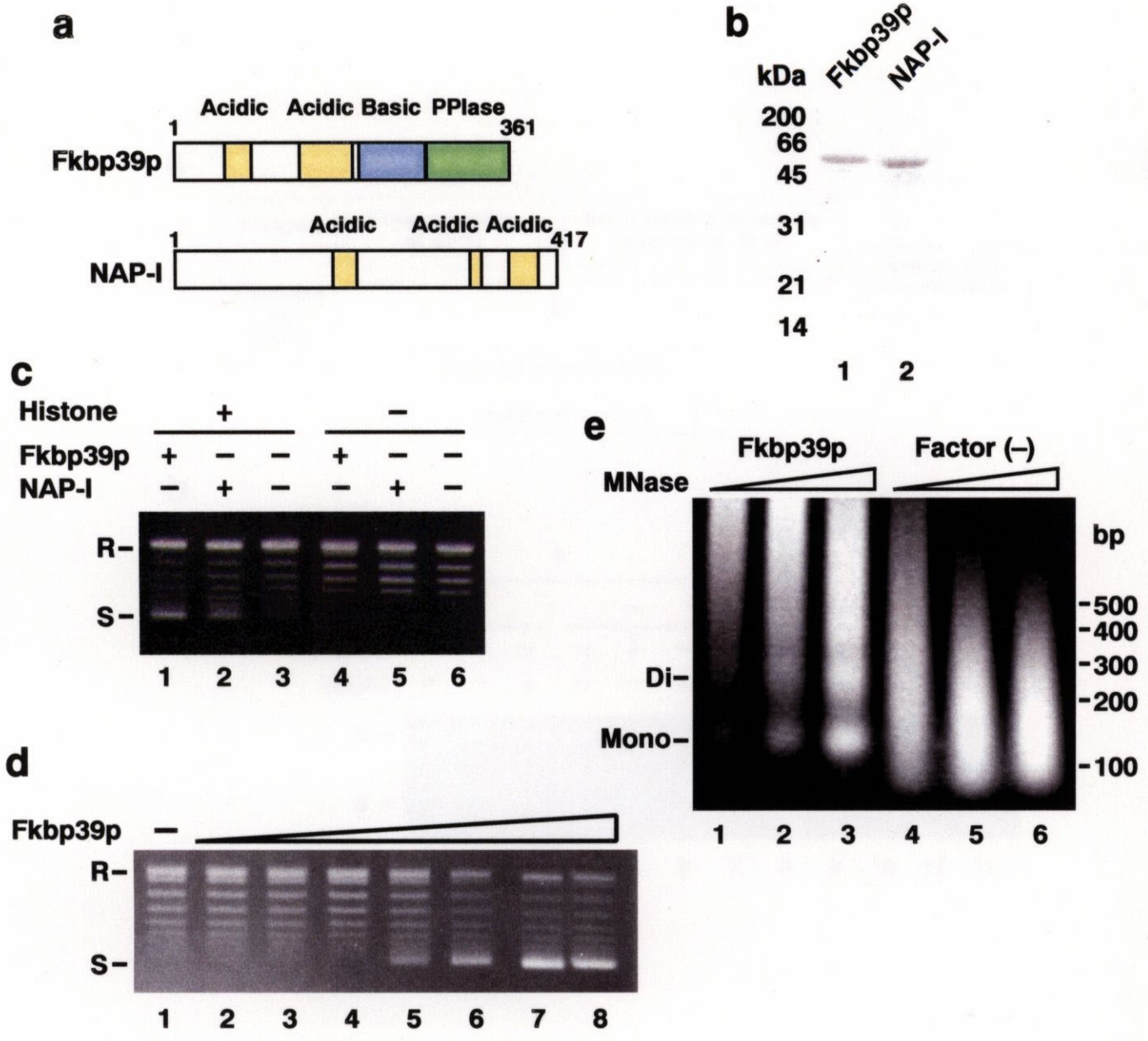


Figure 8: Histone chaperone activity of a nuclear FKBP PPIase.
(**a**) Comparison of the primary structures of SpFkbp39p and NAP-I. Numbers indicate amino acid positions. The acidic/basic regions of SpFkbp39p were originally described by Himukai et al.8 (**b**) Preparation of SpFkbp39p and NAP-I proteins. Proteins (0.2 μg) were analyzed by 12% SDS-polyacrylamide gel electrophoresis and stained with Coomassie brilliant blue. Lanes 1, SpFkbp39p; lane 2, NAP-I. (**c**) Histone chaperone activity as determined by the supercoiling assay. Prior to the assembly reaction, SpFkbp39p or NAP-I was incubated with histones for 1 min at 30°C. After the reaction, the DNA was extracted and analyzed by 1 % agarose gel electrophoresis. Lanes 1 and 4 show the histone chaperone activity of SpFkbp39p (7 pmol). Lanes 3 and 6 show reactions with buffer alone. Lanes 2 and 5 show the histone chaperone activity of NAP-I (7 pmol), which serves as a positive control. Lanes 1-3, reactions in the presence of core histones (4 pmol); lanes 4-6, no core histones. R, relaxed, and S, supercoiled DNA. (**d**) Determination of the stoichiometric relationship of SpFkbp39p to core histones in the histone chaperone reaction. Supercoiling assays were carried out with 0, 0.24, 0.48, 0.96, 1.9, 3.9, 7.7 and 15 pmol (lanes 1–8, respectively) SpFkbp39p and 4 pmol histones. R, relaxed, and S, supercoiled DNA. (**e**) Histone chaperone activity as determined by the micrococcal nuclease (MNase) assay. The amounts of MNase used were 1.3 U (lanes 1 and 4), 2.5 U (lanes 2 and 5) and 5.0 U (lanes 3 and 6). Lanes 1-3, a nucleosome ladder detected in the presence of SpFkbp39p (16 pmol); lanes 4-6, the pattern observed for the control reaction with buffer alone. Mono- and di- indicate mono- and di- nucleosomes, respectively.

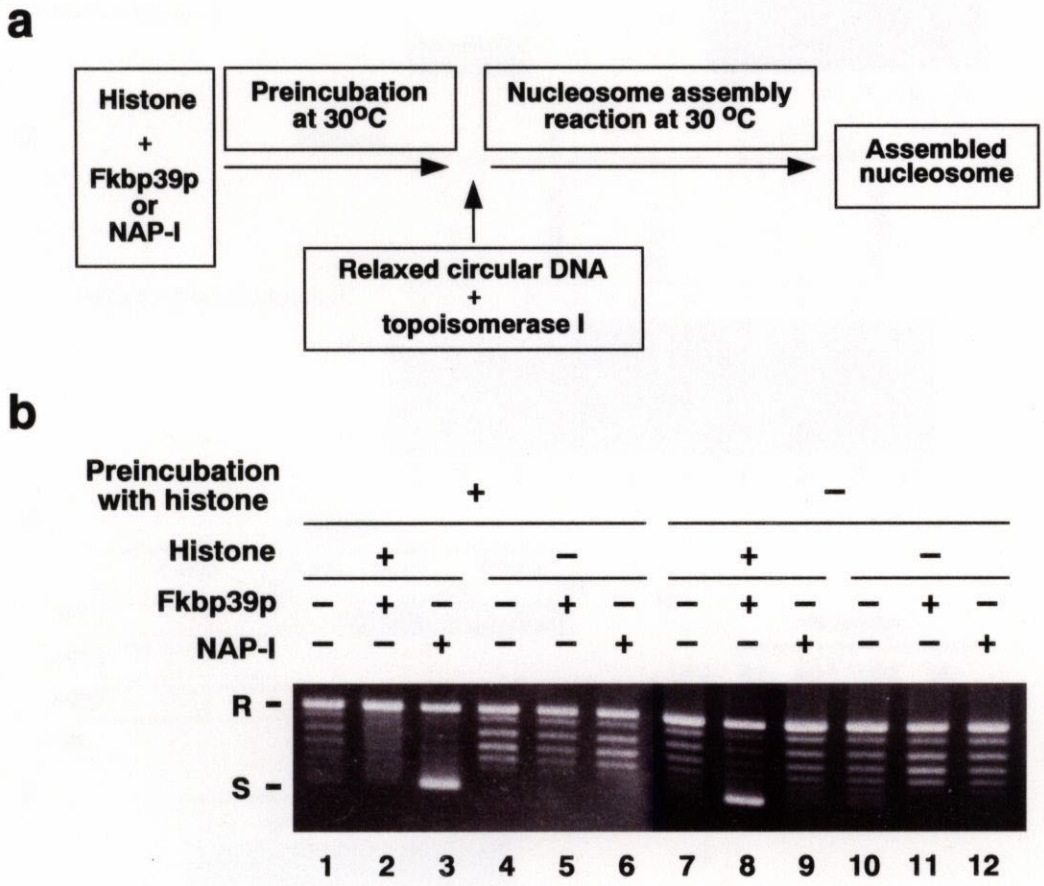


Figure 9: Difference between the histone chaperone reactions promoted by nuclear FKBP and the classical histone chaperone NAP-I.

(**a**) Schematic depiction of the histone chaperone reactions. Core histones and histone chaperones were mixed and incubated for 30 min at 30 °C (pre-incubation step). Relaxed circular DNA and topoisomerase I were then added to the mixture and the incubation was continued at 30 °C to produce assembled nucleosomes. (**b**) The assembly of the nucleosomes by NAP-I, but not by SpFkbp39p, depends on prior incubation with histones. The supercoiling assay was performed essentially as described in Figure 1c. Lanes (1, 4, 7, 10), (2, 5, 8, 11), and (3, 6, 9, 12) represent the results of histone chaperone reactions with buffer alone, SpFkbp39p, and NAP-I, respectively. Lanes 1-6 and 7-12 indicate reactions with (+) and without (-) prior incubation with histones, respectively. Lanes 1-3, 7-9 and 4-6, 10-12 indicate reactions in the presence and absence of core histones, respectively. R, relaxed and S, supercoiled DNA.

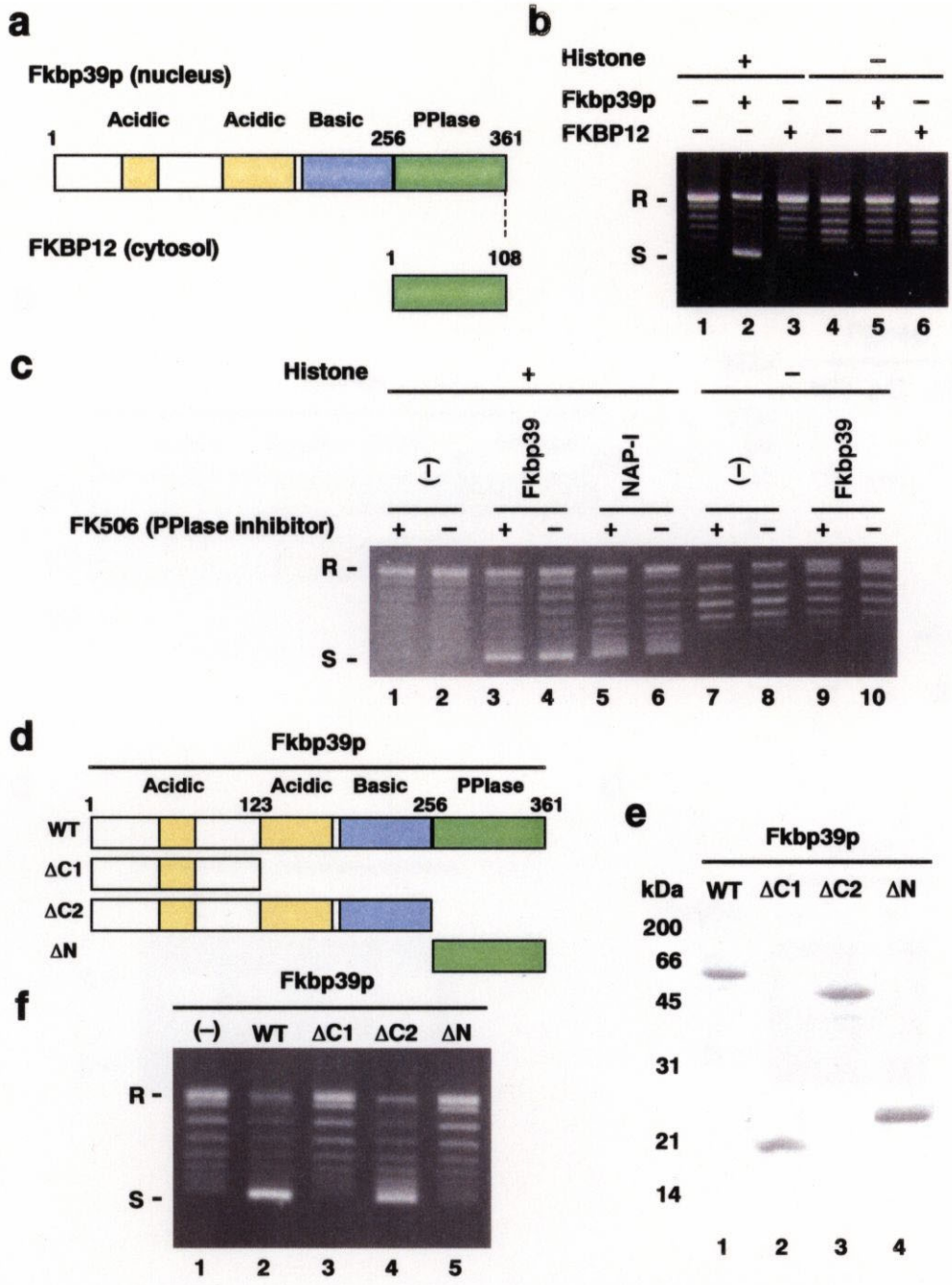


Figure 10: The histone chaperone domain is distinct from the PPIase domain.
(**a**) Comparison of the primary structures of SpFkbp39p (nucleus-specific type) and FKBP12 (cytosol-specific type). Nuclear FKBP has a long N-terminal domain in addition to the PPIase domain, while cytosolic FKBP12 does not. Numbers on the boxes indicate amino acid positions. The subdomains in the N-terminal domain of SpFkbp39p are as described8. (**b**) Nuclear FKBP, but not cytosolic FKBP, has histone chaperone activity. Lanes (1-3) and (4-6) show reactions in the presence and absence of histones (4 pmol), respectively. Lanes (1, 4), (2, 5) and (3, 6) show control reactions with buffer alone, SpFkbp39pb (nuclear type; 7 pmol) and FKBP12 (cytosolic type; 7 pmol), respectively. R, relaxed and S, supercoiled DNA. (**c**) Effect of the PPIase inhibitor FK506 on the histone chaperone activity of SpFkbp39p. Lanes (1-6) and (7-10) show reactions in the presence and absence of histones (4 pmol), respectively. Lanes (1, 2, 7, 8), (3, 4, 9, 10) and (5, 6) show control reactions with buffer alone, SpFkbp39p (7 pmol) and NAP-I (7 pmol), respectively. Odd- and even- numbered lanes show reactions in the presence and absence of FK506 (100 pmol), respectively. R, relaxed and S, supercoiled DNA. (**d**) Schematic structures of the WT and truncated SpFkbp39 proteins. ΔN, ΔC1 and ΔC2 indicate the C-terminal PPIase, the N-terminal (1-123 residues) and (1-256 residues) domains, respectively. The number of amino acids in each segment is indicated on the box. (**e**) Preparation of WT and truncated proteins. Proteins (0.2 μg) indicated in the panel were analyzed by 12 % SDS-polyacrylamide gel electrophoresis and stained with Coomassie brilliant blue. Lanes 1, 2, 3 and 4 represent the purified WT, ΔC1, ΔC2 and ΔN proteins, respectively. (**f**) Histone chaperone activity of WT and mutant proteins. The supercoiling assay was performed under the same conditions as in Figure 1c. Lanes 1, 2, 3, 4 and 5 show histone chaperone reactions with buffer alone, WT, ΔC1, ΔC2 and ΔN proteins (7 pmol), respectively. R, relaxed and S, supercoiled DNA.

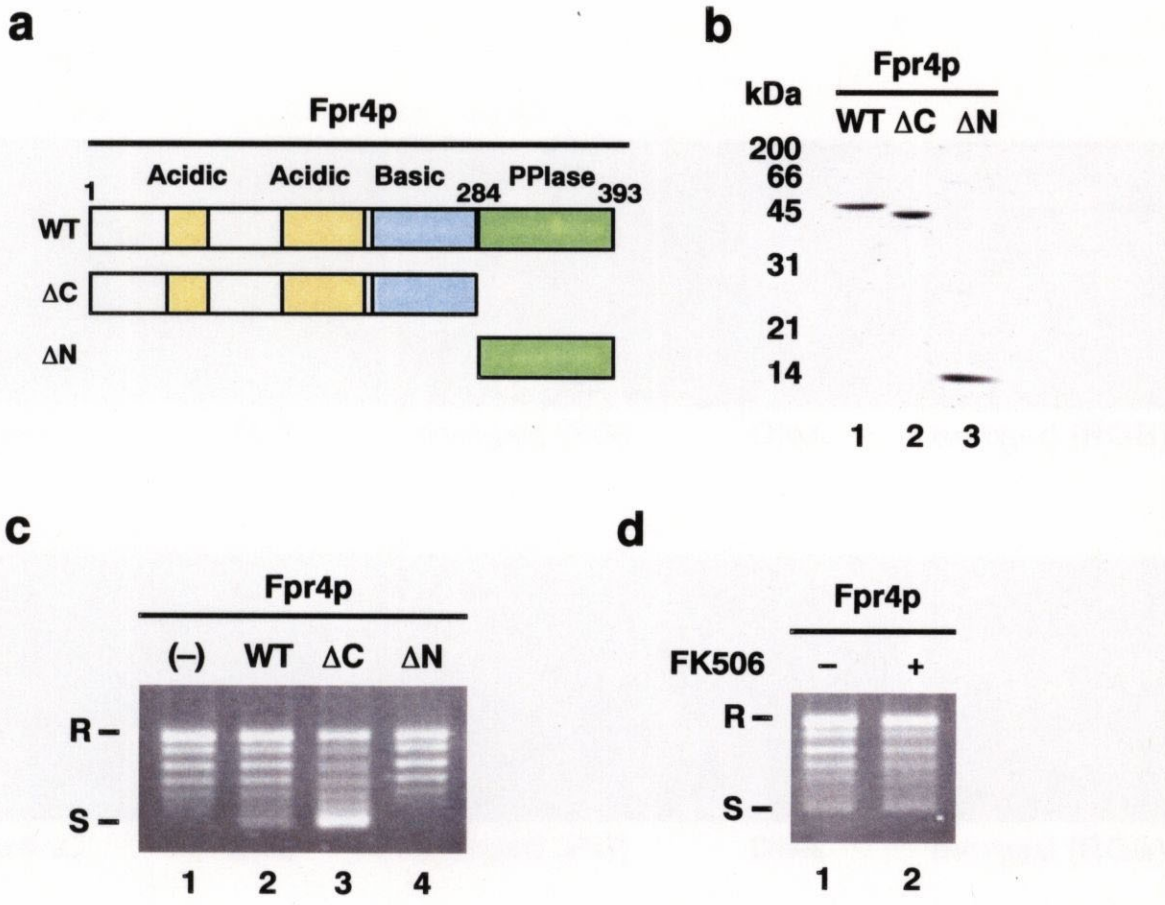


Figure 11: Conservation of the histone chaperone activities of nuclear FKBPs.
(**a**) Scheme of WT and truncated Fpr4 proteins. ΔN and ΔC are the C-terminal PPIase and the N-terminal domains, respectively. The number of amino acids in each segment is indicated on the box. (**b**) Purity of WT and truncated Fpr4 proteins. Proteins (0.2 μg) were analyzed by 12 % SDS-polyacrylamide gels and stained by Coomassie brilliant blue. Lanes 1, 2 and 3 represent the purified WT, ΔC and ΔN proteins, respectively. (**c**) Histone chaperone activities are conserved between the *S. cerevisiae* and *S. pombe* nuclear FKBPs. After the nucleosome assembly reaction, DNA was extracted and analyzed by 1 % agarose gel electrophoresis. Lanes 1, 2, 3 and 4 show reactions with buffer alone, Fpr4 WT, ΔC and ΔN proteins (7 pmol), respectively. R, relaxed and S, supercoiled DNA. (**d**) Conserved effect of the PPIase inhibitor FK506 on the histone chaperone activity by *S. cerevisiae* nuclear FKBP. Lanes 1 and 2 show reactions in the presence and absence of histones (4 pmol), respectively. R, relaxed and S, supercoiled DNA.