STUDIES ON THE INTERACTION OF FKBP65, A PUTATIVE MOLECULAR CHAPERONE, WITH TROPOELASTIN AND AN ELASTIN MODEL POLYPEPTIDE

# STUDIES ON THE INTERACTION OF FKBP65, A PUTATIVE MOLECULAR CHAPERONE, WITH TROPOELASTIN AND AN ELASTIN MODEL POLYPEPTIDE

By

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TITLE: Studies on the interaction of FKBP65, a putative molecular chaperone,

with tropoelastin and an elastin model polypeptide

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#### ABSTRACT

FKBP65 is a 65 kDa FK-506 binding protein containing 4 putative peptidyl prolyl isomerase (PPIase) domains, whose expression level parallels that of tropoelastin, the soluble precursor of elastin. Studies from other laboratories have established that FKBP65 associates with tropoelastin (TE) in the endoplasmic reticulum (ER) and dissociates from TE before reaching the Golgi apparatus (Patterson et al., 2000). TE contains 12% proline residues, which are often found in VPGVG repeats, and it has been suggested that these repeats form  $\beta$ -turns and subsequently  $\beta$ -spirals (Urry et al., 1992). The formation of the  $\beta$ -spiral is thought to be essential to endow the elastic properties of the elastin fibers. In order to form a  $\beta$ -turn, the proline residue at position 2 of the VPGVG sequence must be in trans conformation (Urry et al., 1995). Therefore, it was hypothesized by Davis and coworkers (Davis et al., 1998) that FKBP65, as a PPIase, may play an important role in the folding of tropoelastin by enhancing the formation of  $\beta$ -turns in the ER, and thus elastic fiber formation. In the present study we have studied the coacervation (a reversible, temperature-dependent, self association process) of TE and recombinant elastin model polypeptide, EP4, in the absence or presence of recombinant FKBP65 (rFKBP65). rFKBP65 was shown to enhance the coacervation process of TE, by lowering the coacervation temperature  $(T_c)$  and increasing the overall extent of coacervation. In the kinetic study of coacervation of TE at a constant temperature, rFKBP65 increased both the initial rate of the coacervation process and the overall extent of coacervation. These effects are specific to rFKBP65, as FKBP12 has no effect on the

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coacervation process. Rapamycin, an inhibitor of the PPIase activity of FK-506 binding proteins, did not alter rFKBP65's effect on TE coacervation.

In contrast to TE, rFKBP65 affected the coacervation process of EP4 by increasing the  $T_c$ , and by enhancing the dissociation of coacervates when temperature is decreased. Once again, these effects are specific to rFKBP65, as FKBP12 and BSA were shown to have no effect on the coacervation of EP4. The effect of small pH changes on rFKBP65 was also investigated, and it was found that lowering the pH from 7.5 to 6.0 had no effect on rFKBP65's secondary structure or coacervation-altering activity.

In summary, this study, along with an earlier study from this laboratory, has shown that FKBP65 affects the coacervation process of TE. In addition, the coacervation process of an elastin model polypeptide, EP4, is also modulated by FKBP65. However, the mechanism of these effects remains unclear. Nevertheless, along with the data established by other laboratories, FKBP65 does appear to be a strong candidate as a molecular chaperone for tropoelastin, and may play an important role in the elastogenesis process.

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## **ABBREVIATIONS**

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ATP	- adenosine triphosphate
AU	- absorbance unit
BiP	- immunoglobulin heavy chain binding protein
BSA	- bovine serum albumin
CBD	- chitin binding domain
CD	- circular dichroism
DTT	- dithiothreitol
EBP	- elastin-binding protein
EDTA	- ethylenediaminetetraacetic acid
ECM	- extracellular matrix
EP4	- elastin model polypeptide,
	EP 20-21-23-24-21-23-24-21-23-24-21-23-24
ER	- endoplasmic reticulum
FKBP	- FK-506 binding protein
GuHCl	- guanidine-hydrochloride
HPLC	- high performance liquid chromatography
IPTG	- isopropyl-α-D-thiogalactopyranoside
kDa	- kilodalton
LB	- Luria-Bertani medium
MWCO	- molecular weight cut off
OD	- optical density

PPIase	- peptidyl-prolyl-cis-trans-isomerase
SEC	- size exclusion chromatography
SDS-PAGE	- sodium dodecyl sulfate polyacrylamide gel electrophoresis
T <sub>c</sub>	- coacervation temperature
ТЕ	- Tropoelastin
T <sub>m</sub>	- melting temperature

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#### **CHAPTER 1: INTRODUCTION**

#### 1.1 Elastin

Elastic fibers are the most complex structures of the extracellular matrix (ECM). They provide extensibility and recoil in many tissues such as lung, skin, and blood vessels. These elastic fibers are composed of two distinct components, elastin and a 10-12nm microfibrillar component made up of fibrillin and several related proteins (Ross and Bornstein, 1969). Elastin is the predominant protein of mature elastic fibers accounting for over 90% of their dry weight and responsible for the endowment of elasticity to the fibers. This molecule is an extremely hydrophobic protein and, in its cross-linked form, is highly insoluble (Partridge, 1962). Mutations in the elastin gene impair the normal assembly of elastin precursors into fibers, and lead to inherited diseases such as supravalvular aortic stenosis and Williams-Beuren syndrome (Lowery et al., 1995).

#### **1.1.1 Tropoelastin**

The mechanism of elastogenesis is still poorly understood. Elastin is synthesized and assembled from its soluble precursor, tropoelastin (TE). There is one TE gene present in the mammalian genome (Olliver et al., 1987). The human gene for TE contains 34 exons, which encode for alternating hydrophobic and hydrophilic domains (Figure 1.1). The hydrophilic domains in TE are enriched in Lys and Ala, which are involved in cross-linking and usually occur in repeats such as AAAKAAKAA. The hydrophobic domains are enriched in Gly, Pro, and Val, which often exist in repeats such as GGVP, VPGVG and GVGVAP (Bashir et al., 1989). The expression of TE is tightly regulated, with the highest expression levels occurring during early development, such that it is only



**Figure 1.1. cDNA Structure of Human TE.** The TE gene consists of 34 exons that encode for alternating hydrophobic (white) and hydrophilic (black) domains. Exons were believed to alternate splicing are indicated with an arrow (modified from Weiss et al., 1998).

expressed within a limited time period (Parks et al., 1988). TE contains a hydrophobic signal sequence which targets the molecule to the rough Endoplasmic reticulum (ER) (Saunders and Grant, 1984). TE is then secreted into the lumen of the ER where the signal sequence is cleaved. TE is synthesized with very few translational modifications and there is no evidence of glycosylation. Although some hydroxylation of Pro residues by prolyl hydroxylase does occur, it is believed to be not essential as inhibition of the hydroxylase does not affect TE secretion (Weiss et al., 1998 and Rosenbloom et al., 1976).

There are a few protein candidates that have been found to interact with TE. An elastin-binding protein (EBP) has been suggested as a molecular chaperone for TE. It is believed to play a role in the intracellular transfer of TE and may present TE to the ECM (Hinek and Rabinovitch, 1994 & Mecham et al., 1991). In addition, Davis et al. (1998) showed that TE interacts with ER-localized proteins, BiP and FKBP65. The complex of FKBP65 and TE was found to dissociate before reaching the Golgi apparatus (Patterson et al., 2000). Thus, FKBP65 was hypothesized to be a molecular chaperone for TE. A former student in the lab used *in vitro* assays to show that FKBP65 has enhancement effects on the coacervation process of TE (Bates, 2003). However, the mechanism of the effects and the exact role of FKBP65 in TE synthesis remain unclear.

#### **1.1.2 Structure of Tropoelastin and Coacervation**

Unlike other proteins, TE becomes more structurally ordered with an increase in temperature. This inverse temperature transition is called coacervation and was first demonstrated by Urry et al. in 1969 using circular dichroism (CD), showing that a

pentapeptide, VPGVG, which repeats 11 times in the central region of TE, is converted from a mostly random coil state to a  $\beta$ -like structure upon increasing temperature. Urry suggested that TE forms repeated type-II  $\beta$ -turns by forming a hydrogen bond between the C-O of Val<sup>1</sup> and the N-H of Val<sup>4</sup>. In order to form this bond, the peptidyl-prolyl bond at position 2 must be in the *trans* conformation (Urry et al., 1995) (Figure 1.2). The repeated type-II  $\beta$ -turns may then stack into a  $\beta$ -spiral. The formation of a  $\beta$ -spiral was thought to be essential for conferring elastic mechanical properties to elastic tissues (Patterson et al., 2000). Later in 1983, Bressan et al. showed a disorder-to-order conversion of TE as a result of coacervation using electron microscopy. However, Urry's hypothesis of the formation of  $\beta$ -turns and subsequent stacking into  $\beta$ -spirals has still not been proven.

The process of coacervation is thought to be an essential step to align TE prior to crosslinking by lysyl oxidase (Urry, 1978). Coacervation was shown to be a result of interactions between the hydrophobic domains of TE (Urry, 1992). It has also been shown that the isolated hydrophilic domains do not undergo coacervation (Keeley et al., 2003). In *vitro*, coacervation is usually induced by an increase in temperature, and the change in turbidity can be monitored spectrophotometrically. The coacervation process of TE is reversible by a decrease in temperature. To date, many in *vitro* studies have been done on the coacervation process of TE and TE derivatives, such as EPs (elastin model polypeptides) or ELPs (elastin-like polypeptides). Both EPs and ELPs have been shown to coacervate in a manner similar to TE, although, depending on the architecture of the polypeptide, the coacervation process of some polypeptides is not completely reversible.



**Figure 1.2.** Possible hydrogen bond formation in VPGVG peptide. Left: with Pro<sup>2</sup> in the *trans* conformation, hydrogen bond (dashed line) formation is possible between the C-O of Val<sup>1</sup> and the N-H of Val<sup>4</sup>; Right: with Pro<sup>2</sup> in the *cis* conformation, a hydrogen bond is unable to form. Red: oxygen atom; dark blue: nitrogen atom, and light blue: hydrogen atom; the yellow arrows indicate the positions of C-O of Val<sup>1</sup> and N-H of Val<sup>4</sup>.

However, these elastin model peptides indeed provide a very convenient and informative system to study the coacervation of TE.

#### **1.2 Immunophilins**

Immunophilins are a class of highly conserved intracellular receptors that can be divided into three subclasses based on their specific binding to, and inhibition by, different immunosuppressant drugs. The subclasses (and their inhibitors) are FK-506 binding protein (FK-506/rapamycin (an analog of FK-506)), cyclophilin (cyclosporine A) and parvulin (juglone) (Shireiber, 1991; Gothel et al., 1999). A characteristic that is shared among all immunophilins is peptidyl-prolyl *cis-trans* isomerase (PPIase) activity. PPIases catalyze the interconversion of the *cis-* and *trans-* rotamers of the peptidyl-prolyl amide bond of peptide and protein substrates (Figure 1.3), and this rotamase activity is potently inhibited upon drug binding.

The catalysis of peptidyl-prolyl *cis-trans* isomerization is believed to be important in the folding of proteins to their native conformation, as it has been shown that the *cistrans* interconversion of the peptidyl-prolyl bond is a rate-limiting step in protein folding (Kiefhaber et al., 1990). Thus, PPIases could possibly function as molecular chaperones for protein folding. In addition, other studies have suggested that PPIases may have various roles in processes other than protein folding, such as signal transduction, trafficking, assembly and cell cycle regulation (Patterson et al., 2000; Gothel et al., 1999). However, there is no evidence regarding the importance of PPIase activity in *vivo*.

#### 1.2.1 FKBP12

FKBP12 is the prototypical FKBP family member, having been first identified in

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1989 (Harding et al., 1989). It is the most well characterized protein in the family. FKBP12 has only one PPIase domain and those amino acids that form its hydrophobic binding pocket are conserved among all known FKBP members. FKBP12 has been shown to have a relatively high PPIase activity when compared to other FKBP members. and its PPIase activity can be inhibited by both FK-506 and rapamycin (Schreiber et al., 1991). Structural studies of the FKBP-drug complexes have suggested that the ketone carbonyl adjacent to the homopropyl amide bond of FK-506 and rapamycin is a mimic of the amide carbonyl of a peptide substrate. This structural similarity to the substrate is believed to be the source of the inhibitory activity of these drugs (Shireiber, 1991; Gothel et al., 1999). When FKBP12 is bound to its specific immunosuppressant drugs, the PPIase activity of FKBP12 is inhibited. However, this inhibition of PPIase activity is unrelated to the immunosuppression. Instead, the immunosuppression results from the immunophilin-immunosuppressant drug complex interfering with the essential signal transduction pathways for T cell growth and differentiation (Shireiber and Crabtree, 1992; Galat, 1999; Gothel et al., 1999). The complex of FKBP12-FK506 inhibits Ca<sup>2+</sup> dependent signal transduction by binding to calcineurin, a phosphatase that is essential for early gene transcription events and T-cell growth and differentiation (Shireiber and Crabtree, 1992; Dumont et al., 1990). On the other hand, the FKBP12-rapamycin complex interferes with Ca<sup>2+</sup> independent, IL-2 driven T-cell proliferation (Dumont et al., 1990). Since these interactions are independent of the PPIase activity of FKBP12, it is not clear what biological relevance PPIase activity has.

#### 1.2.2 FKBP65

To date, more than 20 FKBP members (including both eukaryotic and prokaryotic) have been identified, isolated, and sequenced. They have been named according to their molecular masses in kilodalton (kDa), for example: FKBP12, FKBP13, FKBP25, FKBP52 and FKBP65. FKBP65 is a relatively new member of the family. Murine FKBP65 was first cloned by Coss et al. in 1995, and its DNA sequence was found to have homology with other members of FKBPs. FKBP65 has 46% sequence homology with FKBP12, 43% with FKBP13, 35% with FKBP25, and 26% with FKBP52 (Gothel et al., 1999). However, FKBP65 also has its own distinct structural architecture. FKBP65 has 4 PPIase signature domains that share sequence homology to the PPIase domain of FKBP12, a 33 amino acid N-terminal signal sequence, 2 potential Ca<sup>2+</sup> binding EF hand motifs, and an ER retention sequence at the C-terminus (Coss et al., 1995). Studies on FKBP65 have localized it to the lumen of the ER and have determined that it is glycosylated, phosphorylated, and displays PPIase activity (Coss et al., 1995; Zeng et al., 1998). These studies also showed that FKBP65 is expressed in brain, heart, kidney, spleen, testis, and strongly expressed in vascular and lung tissues (Coss et al., 1995; Patterson et al., 2000). The expression of FKBP65 in these areas was found to follow a developmental pattern, with strong expression in early embryonic stages and little or none present in adult tissues (Patterson et al., 2000).

Although some of the characteristics of FKBP65 have been investigated, the biological function of this ER-localized protein remains to be identified. One suggested function for FKBP65 was to form complexes with cytosolic serine/threonine kinase c-

Raf-1 in association with Hsp90 (Coss et al., 1998). However, later in 1998, Davis et al. identified FKBP65 as an ER-localized protein that interacts with TE making the suggested presence of FKBP65 in the cytosol unlikely. By using immunofluorescence, Davis et al. showed that FKBP65 associates with TE in the ER, and dissociates before reaching the Golgi apparatus (Davis et al., 1998; Patterson et al., 2000). Davis et al. hypothesized that FKBP65 is a molecular chaperone for TE based on the following observations: 1) the *cis-trans* interconversion of the peptidyl-prolyl bond is a rate-limiting step in protein folding; 2) TE contains 12% proline residues within its sequence, and FKBP65 may have a role in catalyzing the isomerization of these residues; 3) as suggested by Urry et al., the Val-Pro-Gly-Val-Gly repeats within the TE sequence can form into type-II  $\beta$ -turns if, and only if, the proline residue is in the trans conformation, which may require *cis-trans* interconversion by a PPIase; 4) expression of TE follows the same developmental pattern as FKBP65, which expresses highly only during the embryonic stage; and, 5) FKBP13, another ER-localized FKBP family member has been proposed to function as a molecular chaperone for newly synthesized misfolded proteins in the ER (Bush et al., 1994). All of this implies that FKBP65 may play an important role in catalyzing TE folding, trafficking, and elastic fiber formation. In addition, Davis et al. have recently shown that the expression of FKBP65 was reactivated in the lung of adult mice after treatment of bleomycin instillation to reinitiate the production of matrix proteins, with a pattern similar to that observed for TE (Davis et al., 2005). This finding suggests that FKBP65 may play a role in the biosynthesis of tropoelastin. As previously mentioned, it has been shown that FKBP65 enhances the coacervation process of TE

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(Bates, 2003). This effect was further studied using an elastin model polypeptide, and will be discussed later in detail in this thesis.



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## **1.3 Objective of Thesis**

Taking together all of the findings and suggestions from other researchers, such as Urry and Davis, FKBP65 is a strong candidate for playing role as a molecular chaperone for TE. Therefore, the objective of this thesis is to show whether FKBP65 has or possesses any functional characteristics that would indicate its role as a molecular chaperone for TE. This was done using *in vitro* spectrophotometric assays to determine the effects of purified recombinant FKBP65 (rFKBP65) on the coacervation processes of chick aorta TE and an elastin model polypeptide, EP4.

The results shown in this thesis demonstrate that rFKBP65 has significant but different effects on the coacervation processes of TE and EP4. These effects are specific to FKBP65, as FKBP12 and BSA were shown to have no effect on the coacervation process. Thus, FKBP65 seems to act as a molecular chaperone in the coacervation process of TE. However, the mechanisms of these effects remain unclear.

## **CHAPTER 2: MATERIALS AND METHODS**

#### **2.1 General Materials**

General chemical reagents were obtained from Sigma, BioShop, BioRad, or Fisher Scientific. Protein marker, Broad Range (P7701S) used for SDS-PAGE was purchased from New England BioLabs. Ni-NTA beads were purchased from Novagen. Chick aorta Tropoelastin was purchased from the Elastin Products Company. Elastin model polypeptide, EP4, was provided by our collaborator Dr. F. W. Keeley's laboratory (University of Toronto, Canada).

#### 2.2 Commonly used Buffers

Lysis Buffer:	50mM sodium phosphate, pH 8.0, 1% Triton X-100
	0.2% β-mercaptoethanol
Resuspension Buffer:	10mM Tris-HCl, pH 8.0, 6 M Guanidine-
	hydrochloride, 50mM sodium phosphate, 10mM
	Imidazole, 10mM $\beta$ -mercaptoethanol
Ni-NTA Elution Buffer:	10mM Tris-HCl, pH 8.0, 6 M Guanidine-
	hydrochloride, 100mM sodium phosphate, 250mM
	Imidazole, 10mM $\beta$ -mercaptoethanol
Renaturation Buffer:	20mM Tris-HCl, pH 7.5, 100mM NaCl, 10%
	glycerol, 1mM DTT
Q-Sepharose Wash Buffer:	20mM Tris-HCl, pH 7.5, 100mM NaCl, 100µM
	DTT

Q-Sepharose Elution Buffer:	50mM Tris-HCl, pH 7.5, 750mM NaCl, 1mM
	CaCl <sub>2</sub> , 100µM DTT
Coacervation Buffer A:	40mM HEPES, pH 8.0, 150mM NaCl
Coacervation Buffer B:	50mM Tris-HCl, pH 7.5, 750mM NaCl, 1mM
	CaCl <sub>2</sub> , 10% glycerol, 100µM DTT

#### **2.3 General Methods**

SDS PAGE was performed using 15% Laemmli style gels. Samples were prepared in 3X SDS sample buffer (187.5mM Tris-HCl pH 6.8, 6% (w/v) SDS, 30% glycerol, 0.03% bromophenol blue, 10% (w/v) DTT). Gels were stained with coomassie blue (50% ethanol, 10% acetic acid, 0.1% coomassie blue) and destained with 10% acetic acid.

#### 2.4 Overexpression and Purification of rFKBP65 from E. coli

The pET21a plasmid containing the full coding sequence for murine FKBP65 was received as a gift from Dr. Elaine Davis. This construct was transformed into *E.Coli* strain BL21 (DE3). Overexpression using this construct produces full-length rFKBP65 with a His-tag at the C-terminus for purification using Ni-NTA affinity chromatography.

A 1L culture of BL21 (DE3) transformed with the pET21a-FKBP65 construct was grown at 37°C to an  $OD_{600}$ = 0.6, and then induced with 1mM IPTG at 37°C for 3.5 hours. Cells were harvested by centrifugation at 6000 rpm for 20 minutes. The cell pellet was resuspended in 40ml of Lysis Buffer containing 4 tablets of complete EDTA free miniprotease inhibitor (Roche). 40mg of lysozyme was added and the solution was incubated on ice for 1 hour. The cells were then lysed by sonication. The lysate was centrifuged at

12000rpm for 30 minutes and the insoluble pellet was then washed twice with lysis buffer. The insoluble pellet was then resolubilized in Resuspension Buffer, followed by centrifugation at 12000 rpm for 20 minutes for further clearing. The supernatant was then added to 8 ml of equilibrated Ni-NTA beads and incubated at room temperature with agitation for 1 hour, before the beads were packed into a 2.5cm x 10cm column. The column flow through was collected and run on a 15% SDS-PAGE gel to confirm that rFKBP65 had bound to the Ni-NTA column. The Ni-NTA beads were then washed with 20ml of Resuspension Buffer to remove any non-specific bound proteins. Bound rFKBP65 was then eluted with 20ml of Ni-NTA Elution Buffer. Fractions were collected at each step and run on 15% SDS-PAGE gels. Elution fractions containing purified rFKBP65 were identified and pooled together for renaturation. Renaturation was done by stepwise dilutions to lower the concentration of guanidine-hydrochloride (GuHCl) with Renaturation Buffer, as follows:



The renatured but diluted sample of rFKBP65 was then slowly passed through a 1ml Q-Sepharose column in order to further purify the protein and concentrate the sample. The column was washed with Q-Sepharose Wash Buffer, and then eluted with Q-Sepharose Elution Buffer. The eluted fractions were run on a 15% SDS-PAGE to identify fractions containing concentrated sample. Subsequently, the fractions were pooled together and dialyzed into the proper buffer for later experiments.

## 2.5 Circular Dichroism Characterization of rFKBP65

All CD experiments were performed using a Jasco J-600 spectropolarimeter. For the experiments involving temperature changes, the water jacketed cuvette was attached to a Lauda circulating water bath. All of the spectra were corrected for the spectrum of the buffer and converted to mean residue molar ellipticity by the equation:

$$[\theta] = \frac{\theta_{obs} * MW * 0.1}{c * d * N}$$

where,  $\theta_{obs} = \theta_{mdeg}$  (measured by Jasco 600 CD spec.) MW = Molecular weight of protein in dalton c= concentration in mg/ml d = path length of cuvette in cm N = # of peptide bonds (N=586 for rFKBP65)

To obtain the CD spectrum of purified rFKBP65 at different pHs, samples of rFKBP65 were dialyzed into Coacervation Buffer B at either pH 6.0 or 7.5. Each sample was transferred to a 2mm water jacketed cuvette and a CD spectrum was taken at room temperature. The CD spectrum of rFKBP65 at pH 7.5 was further analyzed using the computer program, Continll, which compares the CD spectrum of rFKBP65 to the CD spectra of 43 reference proteins, and determines its secondary structural contents (Johnson et al., 1999; Keiderling et al., 1991; Yang et al., 1986; and Sreerama et al., 1994). As well, the secondary structural content of rFKBP65 was predicted from its amino acid sequence by Protein Structure Prediction Server, PSIPRED, from the internet. PSIPRED incorporates two feed-forward neural networks, which perform analysis on the output obtained from the PSI-Blast (Position-Specific Iterated BLAST). The determined and predicted structural content were then compared to the secondary structural content of rFKBP65 that was purified using the Intein system (Bates, 2003).

#### 2.5.1 Denaturation Profile of rFKBP65 in Coacervation Buffer B

A sample of purified and renatured rFKBP65 in Coacervation Buffer B was transferred to a 2mm pathlength water jacketed cuvette and capped. The cuvette was then connected to a variable temperature water bath, with a temperature probe set at the tube near the outlet of the cuvette. CD spectra were taken at various temperatures from 19°C to 63°C. The temperature profile was then generated by plotting ellipticity at 225nm,  $[\theta]_{225}$ , versus temperature.

#### 2.6 Size Exclusion Chromatography Analysis of rFKBP65

Size exclusion chromatography (SEC) analyses were performed using System Gold<sup>®</sup> HPLC 126NMP Solvent Module (Beckman) attached to a 24ml Superose 12 FPLC gel filtration column (Pharmacia). All solvents used were filtered and degassed. rFKBP65 was prepared in Coacervation Buffer B, which was also used as the mobile solvent for the experiments. For each experiment, 200µl of rFKBP65 or commercially purchased protein standard mixture (BioRad) was injected into the column. The system was set at a flow rate of 0.5ml per minute and the separation of sample was monitored at 280nm. The elution times from the standard proteins were used to produce a standard curve and a linear regression equation. Using the linear equation, the sizes of the peaks from rFKBP65 sample were calculated.

#### 2.7 Mass Spectrometry Analysis of rFKBP65

Samples of rFKBP65 were dialyzed into 4mM ammonium bicarbonate, pH 8.0 containing 50µM DTT and sent for size determination by mass spectrometry. All the samples were concentrated using ultra centrifugation. A 17 kDa band was cut from SDS-

PAGE gel and sent for MS/MS analysis for protein identification. The mass spectrometry analyses were done at the mass spectrometry facility at McMaster University and performed by Dr. J. Wang. The MS/MS analysis for protein identification of the 65 kDa band showed on SDS-PAGE gel was performed at Advanced Protein Technology Center (APTC) at University of Alberta.

#### 2.8 Coacervation Assay

All coacervation assays for TE and Elastin model polypeptide (EP4) were performed using a Cary 300 UV spectrometer attached to a block temperature control unit. All the samples were pre-mixed on ice and appropriate buffer was added to give each mixture a final volume of  $600\mu$ l. The mixed sample was then transferred to a 1 cm quartz cuvette.

## 2.8.1 Varying-Temperature/Constant Temperature Coacervation Assays with 350nM Tropoelastin

Chick tropoelastin (Elastin Products Company) was solubilized in Coacervation Buffer A to a concentration of 0.67 mg/ml. Each assay mixture contained 350nM TE with or without various concentrations of rFKBP65 that was prepared in the same buffer. The varying-temperature coacervation assays started after 10 minutes of incubation at 15°C and the change in turbidity was monitored at 300nm. The temperature was set to increase 1°C per minute from 15°C to 50°C.

Constant temperature coacervation assays were also performed for TE. Sample preparation was same as above, but turbidometric monitoring was begun immediately after the cuvette has placed into the sample chamber, which was preheated to 45°C for 30

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minutes. In order to determine the significance of PPIase activity on the coacervation process, assays with 1.67mM FKBP12 and 350nM TE was done and assays with 2mM rapamycin added into the FKBP65 and TE mixture were done.

#### 2.8.2 Varying-Temperature Coacervation Assay with 25µM EP4

2mg of EP4 was solubilized in Coacervation Buffer B to a concentration of 2.67 mg/ml. Each assay mixture contained 25μM EP4 with or without 2.6μM of rFKBP65 that was prepared in the same buffer. For the negative controls, rFKBP65 was replaced with FKBP12 or BSA. The measurements started after 10 minutes of incubation at 15°C and the change in turbidity was monitored at 400nm. The temperature was set to increase 1°C per minute from 15°C to 55°C. The reverse coacervation experiments were started immediately after the program had reached 55°C. The temperature was then decreased 1°C per minute from 55°C to 15°C, and the change in turbidity was measured at 400nm. For the pH comparison experiments, due to the decrease in thermal stability of rFKBP65 at pH 6.0, the temperature range was changed to 15°C to 43°C.

#### **CHAPTER 3: RESULTS**

#### **3.1 Production of Recombinant FKBP65**

#### 3.1.1 Overexpression of rFKBP65 in E. coli

*E. coli* BL21(DE3) cells transformed with plasmid pET21a containing the coding sequence of murine *fkbp65* were grown at 37°C to an OD<sub>600</sub> of 0.6, and subsequently induced with 1mM IPTG at 37°C for 3.5 hours. Samples of uninduced and induced cells were collected by centrifugation, resuspended in 1x SDS sample buffer, and boiled for 5 minutes. Each sample was then run on a SDS-PAGE gel for comparison. The overexpression of rFKBP65 is shown in Figure 3.1.1.

#### **3.1.2 Purification and Renaturation of rFKBP65**

The induced cells were resuspended in Lysis Buffer and lysed by sonication. The lysate was separated into soluble and insoluble fractions by centrifugation and the pellet was further washed with Lysis buffer. The resuspended solutions were centrifuged again and the supernatants of those washes were also analyzed by a SDS-PAGE to determine if any rFKBP65 was lost during the washes (Figure 3.1.2 A, lane 2-4). The insoluble fraction was then resolubilized with Resuspension Buffer and centrifuged again for further clearing (Figure 3.1.2 A, lane 5). The resulting supernatant was then incubated with 8ml of pre-equilibrated Ni-NTA beads at room temperature for 1 hour with agitation. The beads were then packed into a column, the flow through was collected and the column was further washed with Resuspension Buffer to remove any non-specific binding proteins. rFKBP65 was then finally eluted with Ni-NTA Elution Buffer and fractions collected were analyzed by SDS-PAGE (Figure 3.1.2 B). The rFKBP65 band



Figure 3.1.1. **Overexpression of rFKBP65 in** *E. coli.* SDS-PAGE of overexpression of rFKBP65 run under reducing conditions and stained with Coomassie Brilliant Blue. Lane 1, protein marker, broad range; Lane 2, uninduced cells; Lane 3, cells induced with 1mM IPTG. The arrow indicates the position of rFKBP65.




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seen in SDS-PAGE ran at expected size of ~65 kDa. The band was cut out and sent for MS/MS spectrometry at University of Alberta, which confirmed the identity of the band as murine FKBP65 (Appendix A1).

rFKBP65 eluted from the Ni-NTA column was then used for renaturation by stepwise dilutions with Renaturation Buffer, followed by dialysis to remove the remaining GuHCl. The renatured but diluted rFKBP65 was then passed through an equilibrated Q-Sepharose column for further cleaning and concentrating. The column bound rFKBP65 was washed with Q-Sepharose Wash Buffer, and eluted with Q-Sepharose Elution Buffer (Figure 3.1.3). There were two advantages to use the Q-Sepharose column. It could remove any contaminants with weaker binding to the column, and also concentrate the diluted rFKBP65 following renaturation. Those fractions containing concentrated rFKBP65 were pooled together and dialyzed into the proper buffer for later experiments.





## 3.2 Characterization of rFKBP65

#### **3.2.1 Size Exclusion Chromatography Analysis of rFKBP65**

SEC was used to study the size of purified rFKBP65 and to determine whether rFKBP65 forms higher order oligomers in solution. A commercially purchased standard protein mixture containing five proteins of different sizes was used to calibrate the system. The chromatogram of the standard mixture is shown in Figure 3.2.1. There were 7 peaks in total, with the peaks eluting at 13 minutes and 16 minutes likely consisting of protein aggregates. The identities of the remaining five peaks are listed in Table 3.2.1, along with their molecular weights and elution times. These values were used to generate a standard curve (Figure 3.2.1). The standard curve shows that the molecular weights and the elution times are well correlated, as the value of the square root of the variance (R<sup>2</sup>) is close to 1.0. It should be noted that the elution times for the standard proteins are only different by a few minutes, while the size range of these proteins varies from 1.35 kDa to 670 kDa (Table 3.2.1). This indicates that a small shift in elution time can result in a large variation in calculated size. The resolution does become better for sizes smaller than 17 kDa (Figure 3.2.1, Peaks 4 and 5).

The chromatogram of purified rFKBP65 is shown in Figure 3.2.2. The sample was not pure, as the chromatogram contains four peaks. The peak at ~13 minutes elutes at the expected time of the column void volume, indicating that this peak likely represents protein aggregates. The remaining three peaks are very broad and overlap, eluting continuously over 20 minutes with no clear separation. The molecular weights of each of the three peaks were calculated using the equation obtained from the standard curve

shown in figure 3.2.1. A summary of elution times and calculated molecular weights for each peak is shown in Table 3.2.2. Peak 2 has a calculated molecular weight of  $114 \pm 7$ kDa, which is similar to the size of a rFKBP65 dimer (120 kDa). The expected dimer mass is 120 kDa but not 130 kDa (theoretical dimer mass of rFKBP65), because the monomeric rFKBP65 has a calculated mass of  $60 \pm 8$  kDa by SEC. If rFKBP65 does dimerize in solution, it is unlikely to be caused by disulfide bonding between monomers, since Coacervation Buffer B contains 100µM DTT. Therefore, any dimerization would likely be due to other interactions, such as hydrophobic interactions. Further investigation is required to determine whether rFKBP65 dimerizes in solution. Peak 3 has a calculated molecular weight of  $60 \pm 8$  kDa, which corresponds to the monomeric form of rFKBP65. Peak 4 was calculated to have a size of  $9 \pm 5$  kDa, indicating large variation in the calculated size of this peak. The identity of this peak remains unknown, because no species representing this mass was seen on SDS-PAGE gels during purification or detected by mass spectrometry. However, mass spectrometry analysis of rFKBP65 sample showed that rFKBP65 does degrade in solution. Thus, this  $9 \pm 5$  kDa peak may be a degradation production of rFKBP65. It was very surprising that this  $9 \pm 5$ kDa peak could not be removed by a 50 kDa molecular weight cut off (MWCO) dialysis tube, as peak 4 was still present in the size exclusion chromatogram following this treatment (data not shown). As mentioned before, the resolution of the system was poor for any sizes larger than 17 kDa, which explains the relatively large standard deviations of the calculated molecular weights. The average elution time shown in Table 3.2.2 for each peak is very consistent, with only a slight variation of 0.3 to 0.5 minutes. However,

this small variation in elution time results in a large variation in calculated molecular weight, which may cause the determined molecular weight to be inaccurate. Although this SEC system has limitations due to poor resolution, it still provided useful information on rFKBP65 in solution, such as the possibility of dimerization, and the fact that there is some contamination present in rFKBP65 samples.



**Figure 3.2.1. Size exclusion chromatogram of standard protein mixture.** SEC was done with a 24ml Superose 12 FPLC column, with Coacervation Buffer B as the mobile buffer. A) Chromatogram of proteins in standard protein mixture. See Table 3.2.1 for identity of peaks and elution times. B) Standard curve generated by plotting log MW and the elution time for each protein in the standard mixture.

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Table 3.2.1. Molecular weights and elution times of protein standardschromatographed on a Superose 12 size exclusion column.

Standard protein	Peak	Molecular Weight (Da)	Elution Time (min)
Thyroglobulin	1	670000	17.50
Gamma globulin	2	158000	21.10
Ovalbumin	3	44000	24.00
Myoglobin	4	17000	27.10
Vitamin B-12	5	1350	35.70



**Figure 3.2.2. Size exclusion chromatogram of rFKBP65.** SEC was done with a 24ml Superose 12 FPLC column, with Coacervation Buffer B as the mobile buffer. The four peaks in the chromatogram are labeled 1 through 4.

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 Table 3.2.2. Elution time and calculated molecular weight data for rFKBP65

 sample.\*

	Peak 2	Peak 3	Peak 4
Elution Time (min)	$22.0\pm0.5$	$24.0 \pm 0.5$	$30.8\pm0.3$
Calculated MW (kDa)	$114 \pm 7$	$60 \pm 8$	9 ± 5

\* SEC analyses were performed using System Gold<sup>®</sup> HPLC 126NMP Solvent Module (Beckman) attached to a 24ml Superose 12 FPLC gel filtration column (Pharmacia). The system was set at a flow rate of 0.5 ml per minute and the separation of sample was monitored at 280nm. A standard curve of the elution times versus the log of molecular weights of the standard proteins in dalton was generated and obtained a linear regression equation. Using the linear equation, the sizes of the peaks from rFKBP65 sample were calculated.

## 3.2.2 Mass Spectrometry Analysis of rFKBP65

Samples of rFKBP65 were analyzed with mass spectrometry to determine its molecular weight. A representative mass spectrum is shown in Figure 3.2.3. rFKBP65 has a calculated molecular weight of 65 kDa from its amino acid composition, however, the region surrounding this mass in the mass spectrum contains multiple clusters of peaks that vary from 61 kDa to 75 kDa. This result, like the size exclusion chromatogram shown in the previous section, indicates heterogeneity in the rFKBP65 sample. The cluster of peaks in the mass spectrum could be caused by the presence of other proteins that have similar mass, however this is unlikely since MS/MS analysis of the 65 kDa band cut from a SDS-PAGE gel revealed that FKBP65 was the only protein present (Appendix A1). Thus, the cluster of peaks indicates that there may be various forms of rFKBP65 present in the sample. Mass spectrometry is a highly sensitive tool and there are many factors that can alter the results of protein mass determination, such as oxidation or binding of ion(s). If oxidation occurred at the acidic amino acids, there will be an increase in mass in multiples of 18 Da. Another possible factor is binding of Na<sup>+</sup> ions. rFKBP65 was purified and concentrated using a Q-Sepharose column prior to dialysis into ammonium bicarbonate buffer for mass spectrometry analysis. All of the buffers used for the Q-Sepharose column contain NaCl, including the elution buffer which contains 750mM NaCl. As a result, any salt bound to rFKBP65 that was not removed during dialysis can cause a shift in the mass of rFKBP65 to higher molecular weights in multiples of 22 Da. However, the differences in mass between various peaks are not consistent, thus, the heterogeneity of rFKBP65 may be due to a combination of factors.

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Figure 3.2.3. Mass spectrum of rFKBP65. Samples of rFKBP65 were sent for mass spectrometry analysis. The analysis was done under denatured condition and a representative spectrum is shown. A peak was detected at 17.8 kDa and another cluster of peaks centered around 67.8 kDa.

Further investigation is needed in order to determine the factors that caused the heterogeneity in the rFKBP65 sample.

The species with masses smaller than 65 kDa may be due to degradation of rFKBP65. Those that are only slightly smaller than 65 kDa may be due to loss of one or more amino acid residues. Varying degrees of degradation may cause the multiple clusters of peaks in the mass spectrum that are smaller than 65 kDa. There was also a sharp peak with significant intensity detected at 17.8 kDa. This mass agrees with a thin band that appears on all the SDS-PAGE gels throughout the purification of rFKBP65 that is ~17 kDa (Figure 3.1.2 and 3.1.3). Therefore, this band of 17 kDa was cut out of an SDS-PAGE gel and sent for MS/MS analysis to determine its identity. The result revealed that this 17 kDa band is a degradation product of rFKBP65 by identifying the amino acid sequence TISDMFQMQDR within the 17 kDa fragment (Appendix A2), which is located near the C-terminus of rFKBP65 (Figure 3.2.4). This 17 kDa degradation product most likely contains the C-terminal His-tag and a pI similar to the full-length rFKBP65, as it was retained during purification using Ni-NTA and Q-Sepharose columns. Thus, an amino acid sequence was predicted for this 17.8 kDa degradation product (Figure 3.2.4). There are 2 tyrosine and 1 tryptophan residues in this predicted sequence (Figure 3.2.4), which absorb at 280nm, and cause inaccuracy in determination of the concentration of fulllength rFKBP65 by absorbance at 280nm. Mass spectrometry shows that degradation of rFKBP65 does occur in solution, and supports the idea that the ~33 kDa species detected by mass spectrometry may also be a degradation product of rFKBP65. As with the  $9 \pm 5$ kDa species from SEC, this 17 kDa fragment of rFKBP65 also could not be removed

1 <u>0</u> MFLVGSSSHT	2 <u>0</u> LHRVRILPLL	3 <u>0</u> LLLQTLERGL	4 <u>0</u> GRASPAGAPL	5 <u>0</u> EDVVIERYHI	6 <u>0</u> PRACPREVQM	
7 <u>0</u> gdfvryhyng	8 <u>0</u> TFEDGKKFDS	90 SYDRSTLVAI	10 <u>0</u> VVGVGRLITG	110 MDRGLMGMCV	12 <u>0</u> NERRRLIVPP	
13 <u>0</u> HLGYGSIGVA	14 <u>0</u> GLIPPDATLY	15 <u>0</u> FDVVLLDVWN	16 <u>0</u> KADTVQSTIL	17 <u>0</u> LRPPYCPRMV	18 <u>0</u> QNSDFVRYHY	
19 <u>0</u> NGTLLDGTGF	20 <u>0</u> DNSYSRGGTY	21 <u>0</u> DTYIGSGWLI	22 <u>0</u> KGMDQGLLGM	23 <u>0</u> CPGEKRKIII	24 <u>0</u> PPFLAYGEKG	
25 <u>0</u> YGTVIPPQAS	26 <u>0</u> LVFYVLLLDV	27 <u>0</u> HNPKDTVQLE	28 <u>0</u> TLELPQGCVR	29 <u>0</u> RAVAGDFMRY	30 <u>0</u> HYNGSLMDGT	
31 <u>0</u> lfdssysrnh	32 <u>0</u> TYNTYVGQGY	33 <u>0</u> IIPGMDQGLQ	34 <u>0</u> GACIGERRRI	35 <u>0</u> TVPPHLAYGE	36 <u>0</u> NGTGDKIPGS	
37 <u>0</u> AVLIFDVHVI	38 <u>0</u> DFHNPSDPVE	39 <u>0</u> IKTLSRPPEN	40 <u>0</u> CNETSKIGDF	41 <u>0</u> IRYHYNCSLL	42 <u>0</u> DGTRLFSSHD	
43 <u>0</u> YEAPQEITLG	44 <u>0</u> ANKVIEGLDR	45 <u>0</u> GLQGMCVGER	46 <u>0</u> RQLIVPPHLA	47 <u>0</u> HGENGARGVP	480 GSAVLLFEVE	
490 LVSREDGLPT	500 GYLFVWYQDP	51 <u>0</u> STSLFEDMDL	52 <u>0</u> NKDGEVPPEE	53 <u>0</u> FSSFIKAQVN	540 EGKGRLMPGQ	
55 <u>0</u> DPDK <b>TISDMF</b>	56 <u>0</u> <b>QNQDR</b> NQDGK	57 <u>0</u> ITAEELKLKS	58 <u>0</u> DEDQERVHEE	58 <u>7</u> <u>1ннннн</u>		
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Figure 3.2.4. Predicted amino acid sequence of the 17 kDa degradation product of rFKBP65. The His-tag rFKBP65 contains 587 amino acids including the 6 histidines of the His-tag at the C-terminus. Numbers on top of the sequence indicate amino acid residues, starting from N-terminus (1) to C-terminus (587). The bold sequence (residues 545-555) was the sequence that was identified by MS/MS analysis of the ~17 kDa band cut out from SDS-PAGE. The underlined sequence is the predicted sequence for the 17.8 kDa degradation product detected by mass spectrometry. This predicted sequence has a calculated mass of 17.8 kDa from its amino acid sequence and a pI of 4.93. The aromatic residues which absorb at 280nm were highlighted.

using a 50 kDa MWCO dialysis tube, as it was still detected by mass spectrometry following this treatment (data not shown).

## 3.2.2.1 Concentration Correction for rFKBP65 samples

Initially the concentration of rFKBP65 was determined by measuring the absorbance of the protein solution at 280nm. The absorbance was then used to calculate the concentration using an extinction coefficient estimated from aromatic amino acid content (Gill and von Hippel, 1989). Since both rFKBP65 and the degradation product absorb at 280nm, the concentration of full-length rFKBP65 determined by measuring the absorbance at 280nm is inaccurate due to overestimation. To solve this problem, the concentration of rFKBP65 in which sample was corrected using a factor that was determined by comparison of the intensities of the bands for the full-length and degraded components of the protein on SDS-PAGE. The bands on the SDS-PAGE gel from the Q-Sepharose column were analyzed using the KODAK 40 Image Station to obtain relative intensities. The bands of rFKBP65 and degraded rFKBP65 consistently had relative intensities of approximately 3:1, respectively (Table 3.2.3). This indicates ~75% of the absorbance at 280nm was contributed by rFKBP65 and the remaining 25% was contributed by the degradation product. Thus, the corrected concentration of rFKBP65 is 75% of the concentration calculated using the estimated extinction coefficient. Since both rFKBP65 and the degraded rFKBP65 contribute absorbance at 280nm, CD was instead used to estimate the concentration of rFKBP65. The degradation product does not have a detectable CD signal presumably due to its low concentration. A CD spectrum with a corrected concentration of rFKBP65 in Coacervation Buffer B was used as a bench

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mark spectrum to estimate the concentration of full-length rFKBP65 from other preparations.

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Table 3.2.3. Relative intensities of SDS-PAGE gel bands of rFKBP65 and degraded product of rFKBP65.

	Relati	ve band Intensity		
Batch	rFKBP65	Degraded rFKBP65	rFKBP65 : Degraded rFKBP65	
1	0.76	0.24	3.1 : 1	
2	0.77	0.23	3.3 : 1	
3	0.77	0.23	3.3 : 1	

Average of ratio:  $3.2 \pm 0.1$ 

# 3.3 Secondary Structure of rFKBP65

CD was used to determine the secondary structure of rFKBP65. CD spectrum of rFKBP65 prepared in the Coacervation Buffer B is shown in Figure 3.3.1. The CD spectrum of rFKBP65 was further analyzed using the computer program, Continll, to determine its secondary structural content. As well, the secondary structure of rFKBP65 was predicted from its amino acid sequence by the Protein Structure Prediction Server, PSIPRED, obtainable from the internet. The calculated secondary structural content obtained using Continll and PSIPRED were similar. On the other hand, when compared to the content of the Intein rFKBP65 (Bates, 2003), there is ~10% difference in the content of  $\alpha$ -helix and  $\beta$ -strand. This may due to the presence of the 33 amino acid signaling sequence in the rFKBP65 purified by His-tag method. In general, the compared results are similar in that the content of secondary structure of the full-length rFKBP65 is made up of ~20%  $\alpha$ -helix, ~30%  $\beta$ -stand, ~ 50%  $\beta$ -turns and random coil (Table 3.3.1). The general agreement on the secondary structural content of rFKBP65 obtained by the His-tag and Intein methods indicates rFKBP65 was renatured correctly during the purification process.



Figure 3.3.1. Circular Dichroism spectrum of rFKBP65. Measurements of rFKBP65 in Coacervation Buffer B at pH 7.5, were taken every 0.2 nm from 250 nm to 202nm in a 0.2 cm jacketed cuvette at room temperature ( $25 \pm 1^{\circ}$ C).

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Table 3.3.1. Comparison of the estimated secondary structural contents of rFKBP65 obtained by the His-tag and Intein methods.

rFKBP65 purification method	Analysis Method	α-helix	β-strand	$\beta$ -turns and random coil
Ni-NTA column	Continll	19.7%	32.2%	48.1%
	PSIPRED	18.3%	29.6%	52.0%
Intein method	Continll	8.9%	38.6%	52.2%

## 3.4 Structural Changes in rFKBP65

The rFKBP65 used in the coacervation assay of EP4 involves high temperatures ( $\geq$ 43°C). Thus, the thermal stability of rFKBP65 in the Coacervation Buffer B was first determined by CD. Since rFKBP65 was assayed for its effects on the coacervation process of EP4 at different pHs, CD spectra of rFKBP65 were also taken at pH 7.5 and pH 6.0. The information obtained from these two experiments was used to set the parameters of the varying-temperature coacervation assay using EP4.

#### **3.4.1 Effect of Temperature**

It has shown that rFKBP65 in Coacervation Buffer A is structurally stable up to  $55^{\circ}$ C (Bates, 2003). However, Coacervation Buffer B is a very different buffer in terms of ionic strength, and since ionic strength could have significant effect on the thermal stability of rFKBP65 (See Materials and Methods), the temperature tolerance of rFKBP65 in this buffer was determined (Figure 3.4.1). rFKBP65 in Coacervation Buffer B was determined to have a melting temperature, T<sub>m</sub>, of 45°C. The CD spectrum of rFKBP65 began to change at ~40°C, and the protein was denatured at temperatures higher than 52°C. Thus, the thermal stability of rFKBP65 is found to be very different in Coacervation Buffer B when compared to Coacervation Buffer A.

#### 3.4.2 pH Effect

The CD spectra of rFKBP65 at both pH 7.5 and 6.0 were measured to determine whether pH affects the secondary structure of rFKBP65 (Figure 3.4.2). The CD spectra at these two pH's were virtually identical, which indicates that there is no secondary structural change in rFKBP65 caused by pH change within this range.



Figure 3.4.1. Temperature effect on the secondary structure of rFKBP65 in Coacervation Buffer B. Measurements of rFKBP65 were taken every 0.2nm from 250nm to 202nm in a 0.2cm jacketed cuvette at various temperatures. The mean residue molar ellipticity of the spectra at 225nm,  $[\theta]_{225nm}$ , were plotted versus temperatures in °C. For this experiment, n=2, and the experimental data are indicated by circles and triangles at different temperatures.



Figure 3.4.2. pH effect on the secondary structure of rFKBP65. rFKBP65 was prepared in Coacervation Buffer B at pH 7.5 and pH 6.0. Measurements were taken every 0.2nm from 250nm to 202nm in a 0.2cm jacketed cuvette at room temperature  $(25 \pm 1^{\circ}C)$ .

# **3.5 Functional Characterization of rFKBP65**

## 3.5.1 Effect of rFKBP65 on the Coacervation Behavior of Tropoelastin in vitro

As mentioned before, Bates (2003) showed that rFKBP65 purified by the Intein system enhances the coacervation process of TE. The effect was also shown to be specific to rFKBP65, when compared to FKBP12 and BSA. The rFKBP65 purified from the His-tag method contains an additional 33 amino acid signaling sequence, and underwent denaturation and renaturation during purification. Thus, some of the experiments of coacervation with TE done by Bates (2003) were repeated to determine whether rFKBP65 purified by the His-tag method has the same specific effects on the coacervation characteristics of TE. The concentration of TE used for these repeated experiments is 350nM instead of 700nM which Bates (2003) used. Therefore, only qualitative comparisons can be drawn between these results and Bates's (2003) results. There are two types of coacervation assay with TE, varying-temperature and constant temperature. The varying-temperature coacervation assay provides information on the effect of rFKBP65 on the coacervation temperature (T<sub>c</sub>) and the overall extent of coacervation (final absorbance at 300nm) at high temperature. On the other hand, the constant temperature coacervation assay determines the effect of rFKBP65 on the kinetics of TE coacervation at a fixed temperature.

## 3.5.2 Coacervation of Tropoelastin as Monitored by Temperature Variation

The results of coacervation assays done on 350nM TE with or without various concentrations of rFKBP65 are shown in Figure 3.5.1. The  $T_c$  was defined as the temperature at which the absorbance at 300nm begins to increase (Figure 3.5.1), and the



Figure 3.5.1. Effect of rFKBP65 on coacervation characteristics of TE. A) Samples of 350nM TE (circles); 400nM rFKBP65 (diamonds); 350nM TE and 400nM rFKBP65 (triangles); 350nM TE and 800nM rFKBP65 (squares) were placed in a UV spectrometer attached to a temperature control unit set at 15°C. The temperature was then increased 1°C/min up to 50°C and the change in turbidity was monitored at 300nm. Two independent trials were performed, and representative data is shown. B) Example showing the determination of  $T_c$ .

overall extent of coacervation was defined as the final absorbance at 300nm at 50°C. 350nM TE alone has a  $T_c$  of 37°C and an overall extent of coacervation of 0.01 AU (absorbance unit). In the presence of 400nM rFKBP65, the  $T_c$  was shifted to 31 °C and the overall extent of coacervation was increased to 0.12 AU. These effects are dose dependent, as 800nM of rFKBP65 had an even greater effect on coacervation, lowering the  $T_c$  to 29°C and increasing the overall extent of coacervation to 0.18 AU. Although this experiment was repeated only twice, and only two different concentrations of rFKBP65 were tested, the trend of the effect is the same as Bates (2003) showed with rFKBP65 purified with the Intein method.

# 3.5.3 Kinetics of Coacervation of Tropoelastin using the Constant Temperature Coacervation Assay

Bates (2003) used the constant temperature coacervation assay and showed that rFKBP65 enhances the initial rate and the overall extent of coacervation of TE, and the effect was saturated at a molar ratio of 2:1 rFKBP65 to TE. The results of repeating the constant temperature coacervation assay on 350nM TE at various concentrations of rFKBP65, are shown in Figure 3.5.2. As expected, the results agreed with Bates's (2003) finding that rFKBP65 increased the initial rate of the coacervation process (the slope between 1 and 3 minutes are steeper), and increased the overall extent of coacervation, as the final absorbance at 300nm was higher. In addition, the effect seems to saturate at a molar ratio of 2:1 rFKBP65 to TE, as 1400nM rFKBP65 does not show any further measurable effect when compared to 700nM rFKBP65.





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# 3.5.4 Specificity of the effect of rFKBP65 on Tropoelastin Coacervation

rFKBP65 purified using the His-tag method was proven to have the same effects on the coacervation of TE as the rFKBP65 purified from the Intein method. In subsequent experiments, rFKBP65 was replaced with FKBP12, to determine the specificity of rFKBP65's effect on TE coacervation. Rapamycin was also added to the TE + rFKBP65 assays to study the significance of PPIase activity on rFKBP65's effects. The results are shown in Figure 3.5.3. The results clearly show that the effect of rFKBP65 is specific, as FKBP12 has no measurable effect on the coacervation process of TE, even at a concentration double that of rFKBP65. In addition,  $2\mu$ M rapamycin did not inhibit the effect of rFKBP65 on the coacervation process. These results once again agree with Bates's (2003) results, and taking it all together, rFKBP65 purified from the His-tag method has the same specific effect on the coacervation process of TE as the rFKBP65 purified using the Intein method.



**Figure 3.5.3.** Specific effect of rFKBP65 on the coacervation kinetics of TE. Samples of 350nM TE (triangles), 350nM TE and 700nM rFKBP65 (diamonds); 350nM TE and 1.67µM FKBP12 (squares); 350nM TE and 700nM rFKBP65 and 2µM rapamycin (circles) were placed in a UV spectrometer attached to a temperature control unit set constant at 45°C. The change in turbidity with respect to time at 45°C was monitored at 300nm. Two independent trials were performed, and representative data is shown.

## 3.5.5 Coacervation Characteristics of Elastin Model Polypeptides

Professor Dr. F. W. Keeley and his colleagues at University of Toronto have synthesized and studied polypeptide models of elastin. Unlike most other researchers who studies on polypeptide models of elastin that represent only a specific hydrophobic region of elastin, Dr. Keeley's polypeptides are composed of different combinations of elastin domains, including both hydrophobic and hydrophilic domains. Thus, these synthesized polypeptides better represent the TE molecule. The Keeley lab has collected a tremendous amount of data on these polypeptides, and has made the following important observations. The synthesized polypeptides contain TE like properties, as they are able to undergo coacervation and subsequently can form fiberous materials with an extended period of incubation at high temperature (Keeley et al., 2002 & 2003). The coacervation of these polypeptides is affected by peptide concentration, ionic strength and pH. Increased concentration of peptide, or increased ionic strength, favors the coacervation by lowering the T<sub>c</sub> of the process (Keeley et al., 2001). Polypeptides containing only hydrophilic crosslinking domains do not undergo coacervation, but these domains will influenced T<sub>c</sub> when present in conjunction with hydrophobic domains. The polypeptides do not undergo coacervation either in pure water or under physiological conditions of 37°C with 150mM NaCl (Keeley et al., 2001). Two most well characterized polypeptides are EP2 (EP 20-21-23-24-21-23-24) and EP4 (EP 20-21-23-24-21-23-24-21-23-24-21-23-24), where 20 and 24 are the human elastin hydrophobic domains while 21 and 23 are the cross-linking domains (Figure 3.5.4). Thus, these polypeptides only represent part of the TE molecule, and EP4 was used to further investigate the effects

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**Figure 3.5.4. Structure of elastin model polypeptides, EP2 and EP4.** EP2 and EP4 are two synthetic elastin model polypeptides from Dr. F. W. Keeley's lab. They are composed of a combination of both hydrophobic (domains 20 and 24) and hydrophilic (domains 21 and 23) domains of TE.

of rFKBP65 on the process of coacervation.

As previously mentioned, coacervation is a process whereby TE undergoes a transition change from a disordered state to an ordered state with an increase in temperature, and the coacervation process is reversible when the temperature is decreased (namely reverse coacervation). The decrease in temperature causes the assembled TE oligomers to dissociate, and a decrease in solution turbidity. The experiments using EP4 were done by monitoring both coacervation and reverse coacervation processes to determine the effect of rFKBP65 on these processes. Since EP4 has a sharp transition between uncoacervated and coacervated state, the temperature at which sudden turbidity increase occurs was defined as the  $T_c$ .

# 3.5.6 Effect of rFKBP65 on the Coacervation Process of EP4 Polypeptide

The results of the coacervation (heating) and reverse coacervation (cooling) processes of  $25\mu$ M EP4 with or without 2.6 $\mu$ M rFKBP65 present are shown in Figure 3.5.5. rFKBP65 affected three aspects of the coacervation process of EP4: the T<sub>c</sub>, the dissociation of the coacervates during reverse coacervation, and the final absorbance at 400nm after reverse coacervation is complete. As seen in Figure3.5.5, EP4 alone has a T<sub>c</sub> of 33°C, and the T<sub>c</sub> was increased to 35°C in the presence of rFKBP65. rFKBP65 also affected the reverse coacervation, that the dissociation of EP4 coacervates was thermodynamically favored in the presence of rFKBP65 as indicated by the steeper slope of the reverse coacervation curve. In addition, the final absorbance at 400nm following reverse coacervation was lower in the presence of rFKBP65, and this may indicate that rFKBP65 caused a decrease in size of the coacervates remaining. Taking these three



Temperature, °C

**Figure 3.5.5. Effect of rFKBP65 on coacervation characteristics of elastin model polypeptide, EP4.** As sample of 25µM EP4 alone (red), or 25µM EP4 and 2.6µM rFKBP65 (black), or 2.6µM rFKBP65 alone (green) was placed in a UV spectrometer attached to a temperature control unit set at 15°C. The temperature was then increased by 1°C/min up to 55°C (circles) and then decreased 1°C/min back to 15°C (triangles). The change in turbidity was monitored at 400nm. Three independent trials were performed and the average curve is shown. The standard deviation was also calculated and shown as the error bars.

aspects together, rFKBP65 seems to limit the coacervation process and the final coacervate size of EP4. These results are very different from the enhancement effect seen with TE. However, EP4 is a synthesized peptide and its interaction with rFKBP65 may be different to that of TE.

The coacervation experiments were also done with 2.6µM FKBP12 or 2.6µM BSA instead of rFKBP65, to determine the specificity of the effects caused by rFKBP65. The results are shown in Figures 3.5.6 and 3.5.7. Neither FKBP12 nor BSA had any measurable effect on either the coacervation or reverse coacervation of EP4. Therefore, the effect seen in Figure 3.5.5 on the coacervation process of EP4 was specific to rFKBP65.

As previously mentioned, Davis et al. has shown that rFKBP65 and TE associate in the ER, and that the complex dissociates before reaching the Golgi apparatus. However, it was not clear whether FKBP65 has a role in the Golgi. Thus, coacervation experiments at two pHs were done to determine whether pH changes alter the effects of rFKBP65 on the coacervation process of EP4. In general, the pH of ER is around neutral (~7.4) and the pH of the Golgi is ~6.4 (Kim et al., 1996). Thus, rFKBP65 and EP4 were prepared in either pH 7.5 or 6.0, and the results of the coacervation assays done at these two pHs are shown in Figure 3.5.8. Recall from the CD characterization of rFKBP65 that the CD spectra of rFKBP65 at pH 7.5 and 6.0 are virtually identical. However, rFKBP65 has a decreased in thermal stability at pH 6.0 as  $2.6\mu$ M rFKBP65 begins to aggregate at temperatures higher than 43°C at pH 6.0 (data not shown). Therefore, in the coacervation assays done at different pH values, the temperature was only increased to a maximum of

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43°C, instead of 55°C. Due to the lower temperature, the coacervation of EP4 did not plateau, and became highly reversible, as indicated by the final low absorbance after cooling. This may be why rFKBP65 did not have any significant effect on reverse coacervation in these experiments. The reverse coacervation curves of EP4 with or without rFKBP65 ended at the same absorbance. In addition, the effect of FKBP65 on the dissociation of coacervates during reverse coacervation became less significant. However, the shift in T<sub>c</sub> caused by rFKBP65 remained the same. Although, due to the limitations in these experiments, the effects of rFKBP65 were less apparent, there was still a noticeable difference in coacervation properties when rFKBP65 was present. However, this result is insufficient to provide any information on the role of FKBP65 in the Golgi apparatus.












### **CHAPTER 4: DISCUSSION**

In this work, rFKBP65 was produced from a construct containing the full *fkbp65* gene cloned into pET21a. This construct places a His-tag on the C-terminus of the fulllength FKBP65, which allows for purification using a Ni-NTA column. In contrast, Bates (2003) used a construct with the *fkbp65* gene (minus the N-terminal signal sequence) cloned into pTYB4 vector. This construct produces an rFKBP65-Intein-Chitin binding domain (CBD) fusion protein, which could be purified using chitin beads. The intein-CBD domain was subsequently removed using a reducing agent, leaving behind untagged native rFKBP65. From this point on in the discussion, rFKBP65 produced from the pET21a construct is referred to as "His-tag rFKBP65", and rFKBP65 produced from the pTYB4 construct is referred to as "Intein rFKBP65". Where I do not distinguish between the two, "rFKBP65" refers to the His-tagged version with which the experiments in this study were carried out. There are advantages and disadvantages of producing rFKBP65 from the pET21a construct. His-tag rFKBP65 was insoluble in E. coli, and required long purification procedures, including denaturation and renaturation steps. In contrast, the Intein construct yields soluble rFKBP65 with fewer purification steps, and no extra affinity tag. However, the main advantage of using His-tag rFKBP65 is the higher yield. The yield of rFKBP65 per liter of media was approximately 6 times higher for His-tag rFKBP65 than for Intein rFKBP65 (0.57mg versus 0.12mg). In addition, Intein rFKBP65 preparations were contaminated with the E.coli chaperonin, GroEL. The size and the pI of GroEL are very similar to FKBP65, which makes separation of the two proteins very difficult. However, Bates (2003) had removed GroEL bound to FKBP65 by

washing with a buffer contains ATP while the complex is bound to the chitin column. This is due to that GroEL, but not FKBP65, has an ATP-binding site, and the ATP in the buffer competes with FKBP65 to bind to GroEL. The GroEL dissociated from FKBP65 is thus eluted with the ATP buffer while FKBP65 is still bound to the chitin column. The GroEL removal problem can be avoided by using the His-tag rFKBP65. The His-tag rFKBP65 remains in the insoluble fraction, while GroEL is found in the soluble fraction of the lysate. In addition, the His-tag rFKBP65 was purified under denaturing conditions using affinity chromatography with Ni-NTA beads. Therefore, it is unlikely that GroEL can bind to rFKBP65 during purification. However, His-tag rFKBP65, which affected determination of rFKBP65 concentration. Since the impurities are fragments of rFKBP65, it is unlikely that the effects seen on the coacervation processes of TE and EP4 are caused by a protein other than FKBP65.

The rFKBP65 sample was characterized using several techniques, each of which provides different information on rFKBP65. Firstly, SEC showed that the rFKBP65 sample was not completely pure, since the chromatogram contains four peaks (Figure 3.2.2). The chromatogram of rFKBP65 suggested that it may dimerize in solution, as one of the peaks (Peak 2) has a calculated size of  $114 \pm 7$  kDa, that is relatively similar to the expected size of dimeric rFKBP65 (~120 kDa). The expected mass of dimeric rFKBP65 is 120 kDa but not 130 kDa (theoretical dimer mass of rFKBP65), because the mass for monomeric rFKBP65 was calculated as  $60 \pm 8$  kDa by SEC. rFKBP65 was prepared in buffer containing 100µM DTT, thus any dimerization is unlikely to be due to disulfide

bond formation. However, further investigation is necessary to study the dimerizaton. such as using a technique like dynamic light scattering. Peak 3 had a calculated size of 60  $\pm$  8 kDa, which corresponds to the monomeric form of rFKBP65 (65 kDa). The mass of the Peak 4 in the size exclusion chromatogram was calculated as  $9 \pm 5$  kDa. However, there is no band of that size present in the SDS-PAGE gels throughout purification, and mass spectrometry did not detect any species that represents  $9 \pm 5$  kDa. Thus, the identity of this peak remains unknown. However, mass spectrometry analysis showed that rFKBP65 does degrade in solution, therefore the peak of  $9 \pm 5$  kDa may be a minor degradation product of rFKBP65. The size exclusion system has a poor resolution, as discussed in the Results section, thus the calculated sizes may be inaccurate. Furthermore, the peaks seen in the rFKBP65 sample were broad and continuously eluted over 20 minutes, without any clear separation, and the absorbance for the sample of rFKBP65 was very low (<0.01 AU at 280nm) due to low concentration. These probably contributed to inaccuracy in the calculated sizes, and represents heterogeneity in the sample of rFKBP65. Despite its limitations, SEC was useful to show the presence of the contaminant in the rFKBP65 sample.

Mass spectrometry was also used to determine the molecular weight of rFKBP65. The mass spectrum showed a cluster of peaks centered at 67.9 kDa, and a sharp peak at 17.8 kDa (Figure 3.2.3). The cluster of peaks around 67.9 kDa indicates heterogeneity in the sample, as was also seen with SEC. Masses larger than 65 kDa may be due to binding of ions or oxidations of rFKBP65. As mentioned before, the mass differences between the various peaks in the cluster are inconsistent, thus, the cause of the cluster of peaks

may be due to a combination of factors. On the other hand, masses smaller than 65 kDa may be due to different degrees of degradation, such as loss of one or more amino acid residues. The 17.8 kDa peak identified by mass spectrometry is consistent with a band of  $\sim$ 17 kDa which appears in all SDS-PAGE gels during purification (Figure 3.1.2 and 3.1.3). Thus, this band was cut out of the gel and further analyzed by MS/MS. MS/MS analysis identified an amino acid sequence of TISDMFQMQDR within the 17 kDa fragment, which is located near the C-terminus of rFKBP65 (Figure 3.2.4). Therefore, this 17.8 kDa species is a degradation product of rFKBP65, which most likely contains the C-terminal His-tag and a pI similar to the full-length rFKBP65, as it was retained during purification using Ni-NTA and Q-Sepharose columns. Thus, an amino acid sequence predicted for the 17.8 kDa degradation product (Figure 3.2.4) has a calculated mass of 17.8 kDa and contains a His-tag at the C-terminus and has a pI of 4.93. The pI value is different from the full-length FKBP65 (pI = 5.43). However, all the buffers used throughout Q-Sepharose column purification were at pH 7.5, therefore both the fragment and the full-length rFKBP65 would be negatively charged and would not be separated by Q-Sepharose chromatography. The predicted sequence of the 17.8 kDa rFKBP65 degradation product contains 2 tyrosine and 1 tryptophan residues (Figure 3.2.4), which absorb at 280nm, and have to be taken into account while in determining the concentration of full-length rFKBP65 by absorbance at 280nm. This problem was solved as described in section 3.2.2. Since this degradation product was also present in fractions eluted from the Ni-NTA column (Figure 3.1.2 B), the degradation may be caused by protease cleavage which occurs during the process of cell growth and overexpression of

rFKBP65. There is another species detected by mass spectrometry that has a mass of ~33 kDa (Figure 3.2.3). However, there is no band of that size present in the SDS-PAGE gel, thus, its identity cannot be confirmed. Although both SEC and mass spectrometry showed that the sample of rFKBP65 was impure, rFKBP65 was still shown to have effects on the coacervation processes of TE and EP4.

CD was used to determine the secondary structure spectrum of rFKBP65, and the spectrum was further analyzed by Continll to determine the secondary structural content of rFKBP65. Compared to the secondary structural contents predicted from PSIPRED and obtained from the Intein rFKBP65, there is a ~10% difference in both  $\alpha$ -helical and  $\beta$ -strand contents between Intein rFKBP65 and His-tag rFKBP65. This may due to the presence of the 33 amino acid signal sequence in His-tag rFKBP65 (Table 3.3.1). Nevertheless, the Continll results for His-tag rFKBP65 highly agree with the predicted secondary structure contents from PSIPRED, that rFKBP65 has approximately 20%  $\alpha$ -helix, 30  $\beta$ -strands, and 50%  $\beta$ -turns and random coil. This agreement on the secondary structure contents indicates that His-tag rFKBP65 has folded correctly during renaturation.

CD was also used to study any structural changes in rFKBP65 caused by changes in temperature or pH. rFKBP65 in Coacervation Buffer B, containing 750mM NaCl, was determined to have a  $T_m$  of 45°C. From the denaturation profile at 225nm (Figure 3.4.1), the structure of rFKBP65 began to change at ~40°C, and it was completely denatured at temperatures higher than 52°C. rFKBP65 is seen to have no secondary structural changes between pH 7.5 and 6.0 (Figure 3.4.2). The characterization on the structural tolerances

of FKBP65 was used to set the parameters for the coacervation assays that involved high temperature and pH changes. It should be noted that rFKBP65 was prepared in Coacervation Buffer B, with a salt concentration (750mM NaCl) which is a lot higher than physiological concentration (150mM NaCl).

Since FKBP65 was hypothesized as a molecular chaperone for TE, our aim in this thesis is to investigate their interaction between these proteins by studying the effects of rFKBP65 on the coacervation process of TE. It was already shown by Bates (2003) that Intein rFKBP65 enhances coacervation of TE. However, His-tag rFKBP65 has a small but significantly different sequence from Intein rFKBP65 in having the additional 33 amino acid signaling sequence at the N-terminus, and it was purified under denaturing conditions, with subsequent renaturation. Some of the coacervation assays with TE done by Bates (2003) were repeated to confirm whether His-tag rFKBP65 has the same specific effects on the coacervation characteristics of TE as Intein rFKBP65. Comparisons of the effects of Intein rFKBP65 and His-tag rFKBP65 on the coacervation of TE were made qualitatively, because different concentrations of TE were used (700nM TE versus 350nM TE) which would cause a difference in their coacervation behavior. The varying-temperature coacervation assay showed that His-tag rFKBP65 enhances the coacervation process of TE by decreasing the T<sub>c</sub> and increasing the overall extent of coacervation (Figure 3.5.1). This effect was dose dependent, with increased enhancement seen at higher rFKBP65 concentration. The constant temperature coacervation assay showed that rFKBP65 increased both the initial rate and the overall extent of coacervation, and the effects seem to saturate at a molar ratio of 2:1 rFKBP65 to TE

(Figure 3.5.2). These effects were specific to rFKBP65, as FKBP12 was shown to have no effect on the coacervation process of TE. Besides, Bates (2003) had shown that BSA also has no effect on the coacervation of TE. The presence of excess rapamycin did not inhibit the effects of rFKBP65. This result shows that PPIase may not have a role in the effect of rFKBP65 on the coacervation of TE. All these results agree with Bates's (2003) results obtained using Intein rFKBP65, which indicates that denaturation and renaturation during purification did not affect proper folding or activity of FKBP65.

We have extended the studies on TE-FKBP65 interaction to an important additional collaborative project that exploits the availability of recombinant elastin model polypeptides (EPs) in Dr. F. W. Keeley's laboratory at the University of Toronto. As a first step, the effects of rFKBP65 on the coacervation process were further studied using EP4. EP4 represents a specific region of TE that consists of both hydrophilic and hydrophobic domains of TE (Figure 3.5.4), and it was used to determine if FKBP65 has any effect on its coacervation process. Our data showed that rFKBP65 affected the coacervation process of EP4 by delaying the T<sub>c</sub> by 2°C and that the dissociation of the coacervates was thermodynamically favored in the presence of rFKBP65 during reverse coacervation, thereby maintaining the dissociated coacervates at a smaller size. The effects of rFKBP65 on the coacervation process of EP4 seem to indicate that rFKBP65 was limiting the coacervate size. These effects of rFKBP65 are different from its effects on the coacervation of TE. It should be mentioned that the concentration of EP4 used is a lot higher than TE (25µM EP4 versus 350nM TE), and the conditions (high ionic strength with high concentration of peptide) used for EP4 are sufficient for it to complete the

coacervation (as indicated by the sharp transition and perfect sigmoidal coacervation curve) when compared to TE (a slowly increasing coacervation curve). Considering the results of the coacervation assays with both TE and EP4, it would appear that FKBP65 may enhance the coacervation of TE and also limit the coacervate size. This may have in vivo relevance for transfer of TE to the Golgi apparatus and ECM. However, EP4 is a synthetic peptide that mimics only a specific region of elastin, and it requires different conditions in order to undergo coacervation. Thus, rFKBP65 may interact with, and affect TE and EP4 differently. Neither FKBP12 nor BSA had an effect on the coacervation of EP4, which indicates that the effects seen with rFKBP65 are specific. As mentioned before, FKBP12 is the most well studied member of the FKBP family, and it has been shown to have the highest PPIase activity among the family. Furthermore the FKBP65 mediated coacervation of TE was not altered in the presence of rapamycin. This would suggest that the PPIase activity has no role in the effects of rFKBP65 on the coacervation process. It is unfortunate that the elastin model polypeptides containing proline to glycine mutations do not undergo coacervation, instead the peptides form a fibrillar precipitate resembling amyloid fibrils (Keeley et al., 2003). Otherwise, these peptides would be useful to further study the role of PPIase activity in the coacervation process.

It is not known which domains of TE interact with FKBP65. The effects of rFKBP65 on the coacervation process of EP4 indicate that rFKBP65 may interact with domains 20, 21, 23, or 24 of TE. This can be further studied with other elastin model polypeptides available from Dr. Keeley's lab, such as EP 20-21-23-20-21-23-20 and EP

24-21-23-24-21-23-24, which contain repeats of only one of the hydrophobic domains. EP 21-23, which consists of only the hydrophilic crosslinking domains does not undergo coacervation (Keeley et al, 2001). As an alternative, elastin-like peptide (ELP) that contains repeats of pentapeptide sequence Val-Pro-Gly-Xaa-Gly (where Xaa can be any amino acid except Pro) representing the hydrophobic domain of TE, can be used to determine whether FKBP65 interacts with the crosslinking domains of TE. ELP has been shown to undergoes coacervation, and the coacervation process is highly reversible (Chilkoti et al., 2001). ELP is available in Professor Dr. C. Filipe's laboratory at the department of chemical engineering. The advantage of studying the effects of rFKBP65 on different polypeptides is that these polypeptides represents different regions of the TE molecule, some of which may not contain the binding or interacting region and should not be affected by FKBP65. This provides a good system for determining the domain(s) of TE that FKBP65 interacts with.

A change in pH from 7.5 to 6.0 does not alter the CD spectrum of rFKBP65. rFKBP65 still shifted the  $T_c$  of the coacervation of EP4. However, the thermal stability of FKBP65 was lower at pH 6.0 as rFKBP65 aggregates beyond 43°C. It should be noted that the condition used in the pH experiments on rFKBP65 was not physiological, as the salt concentration was 750mM and the temperature was increased to higher than 37°C. Therefore, the shift in pH from 7.5 to 6.0 which caused a decrease in the thermal stability of FKBP65 is only valid in this condition. These results are in contrast to those on the collagen chaperone, Hsp47, whose secondary structure is altered and whose inhibiting effects on type I collagen fibril formation are diminished when the pH is changed from

7.0 to 6.0 (Thomson and Ananthanarayanan, 2000). Although a change in pH from 7.5 to 6.0, the CD spectrum remains the same and rFKBP65 still shifted the  $T_c$  of the coacervation process of EP4, these results are insufficient to show whether FKBP65 has a role in the Golgi apparatus *in vivo*.

Coss et al. had shown that FKBP65 undergoes post translational modifications such as glycosylation and phosphorylation (Coss et al., 1995). These modifications are lacking in our rFKBP65, which was grown and overexpressed in *E.coli*. However, the fact that our results showed that rFKBP65 produced in *E.coli* affects coacervation of TE and EP4 indicates that the post translational modifications of FKBP65 may not be essential for its effect on the coacervation of TE.

Taking together, the cell biological data of Davis et al., as well as our physical chemical data on the effect of rFKBP65 on the coacervation of TE and EP4, suggest FKBP65 to be a strong candidate for acting as a molecular chaperone for TE. Further studies are necessary to determine the exact role and the mechanism of these effects of FKBP65 on the coacervation process. One of these would be to carry out the constant temperature coacervation assay with EP4, as done for TE, to obtain insights on how FKBP65 affects the initial rate of EP4 coacervation, or investigating whether the effects are dependent on the concentration of rFKBP65. A long-term approach is to use the two types of assay, varying or constant temperature, to further study the interaction of TE KBP65 with other elastin model polypeptides that consist of different domains of TE molecule. In addition, ELP may also be tested to determine if FKBP65 interact with the crosslinking domains of TE. As mentioned previously, we suspected that some of the

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elastin model polypeptides do not contain the interacting domain with FKBP65, and therefore FKBP65 should have no effect on their coacervation process. By testing different polypeptides, we can hope to discover the domain(s) of TE that FKBP65 interact(s) with, and to further understand the mechanism of the effects of FKBP65 on coacervation of TE. Such studies will increase our understanding of the exact role of FKBP65 in the elastogenesis process.

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Result of MS/MS analysis of the 65 kDa band from SDS-PAGE.

# SCIENCE Mascot Search Results

User	: Paul Semchuk					
Email	: paul.semchuk@ualberta.ca					
Search title	: 3p064-fkbd65					
MS data file	: C:\WINNT\Profiles\Micromass\Desktop\3p064-mgf\3p064 fkdb65.mgf					
Database	NCBInr 20031101 (1547988 sequences; 504940068 residues)					
Timestamp	: 7 Nov 2003 at 17:35:53 GMT					
Significant hits	: gi 18034674 65kDa FK506-binding protein [Mus musculus]					
	gi 595990 33 amino acid polypeptide; Ncol site (105) allows clo					

#### **Probability Based Mowse Score**

Ions score is -10\*Log(P), where P is the probability that the observed match is a random event. Individual ions scores > 46 indicate identity or extensive homology (p<0.05). Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.



#### **Peptide Summary Report**

Form	nat As	Peptide Sumr	nary	•						Help
		Significance	threshold p<	0.05	Max. n	umber	of hits	20		
		Standard sco	ring @ Mud	PIT scoring	Ions sc	ore cut	-off 0			Show
		Show pop-up	os @ Suppre	ss pop-ups 🤈	Sort un	assign	ed Decr	easing Sco	ne	🛃 Requ
Selec	gi 180 65kDa Check	Select None 34674 FK506-bind: to include	Mass: 651 Ing protein this hit i	78 Score [Mus muscu. n error tole	F Error : 875 lus] erant se	Quer Quer arch	ant ies mat	ched: 3	0	
	Query	Observed	Mr (expt)	Mr (calc)	Delta	Miss	Score	Expect	Rank	Peptide
P	1	401.2330	800.4515	800.4392	0.0124	0	35	0.88	1	VIEGLDR
14	3	445.1864	888.3581	888.3613	-0.0032	0	40	0,32	1	FDSSYDR
2	4	500.7455	999.4763	999.4695	0.0068	0	(25)	8.1	1	LMPGQDP

P	5	508.7439	1015.4733	1015.4644	0.0089	0	32	1.6	1	LMPGQDP
5	6	509.2429	1016.4713	1016,4563	0.0150	1	28	4.1	1	KFDSSYD
2	B	540.7596	1079.5047	1079.5069	-0.0022	0	35	0.62	1	EVQMGDF
5	9	549.2687	1094.5228	1094.5178	0.0050	0	(32)	1.3	1	MVQNSDF
	10	548.2703	1094.5260	1094.5178	0.0082	0	(13)	1.1e+02	7	MVQNSDF
P	11	548.7621	1095.5097	1095.5018	0.0079	0	(35)	0.67	1	EVQMGDF
F	14	556.2709	1110.5273	1110.5127	0.0146	0	50	0.019	1	MVQNSDF
V	15	560.2800	1118.5455	1118.5203	0.0252	1	61	0.0021	1	LKSDEDQ
4	16	560.2899	1118.5653	1118.5203	0.0449	1	(38)	0.34	1	LKSDEDQ
12	24	585.8751	1169.7356	1169.7131	0.0225	0	62	0.0018	1	STLVAIV
<u>ح</u> ا	25	585.8855	1169.7564	1169.7131	0.0433	0	(12)	1.6e+02	1	STLVAIV
17	30	665.7978	1329.5811	1329.5625	0.0186	0	11	1.7e+02	1	YHYNGTF
17	31	680.9019	1359.7893	1359.7802	0.0091	0	38	0.34	1	ILIPPEL
12	32	680.9076	1359.8007	1359.7802	0.0205	0	(38)	0.36	1	IIIPPFL
17	37	486.8994	1457.6765	1457.6575	0.0190	L	28	2.9	1	YHYNGTF
1	41	790.8895	1579.7645	1579.7405	0.0240	0	40	0.24	1	DGEVPPE
5	42	790.8915	1579.7684	1579.7405	0.0278	0	(23)	11	1	DGEVPPE
2	45	815.9207	1629.8269	1629.8038	0.0231	0	86	4.70-06	1	GGTYDTY
P	50	570.3106	1707.9099	1707.9168	-0.0069	0	47	0.04	1	QLIVPPH
5	51	590.2955	1767.8646	1767.8791	-0.0145	0	(24)	6.4	1	ITVPPHL
P	52	590.3024	1767.8855	1767.8791	0.0064	0	60	0.0017	1	ITVPPHL
শ	54	663.3582	1987.0529	1987.0197	0.0332	0	83	1.10-05	1	ADTVQST
P	55	707.3408	2119.0005	2119.0221	-0.0217	0	(28)	2.2	1	LFSSHDY
7	56	707.3497	2119.0272	2119.0221	0.0051	0	60	0.0014	1	LFSSHDY
F	57	707.3509	2119.0308	2119.0221	0.0086	0	(47)	0.027	1	LFSSHDY
1	5 B	731.9954	2192.9645	2192.9762	-0.0118	0	(41)	0.13	1	YHYNGTL
17	59	732.0034	2192.9884	2192.9762	0.0122	0	81	1.2e-05	1	YHYNGTL

2. <u>gi[595990</u> Mass: 3723 Score: 49 Queries matched: 6 33 amino acid polypeptide; NooI site (105) allows cloning at translation initi Check to include this hit in error tolerant search

	Query	Observed	Mr (expt)	Mr(calc)	Delta	Miss	Score	Expect	Rank	Peptide
1	17	562.2601	1122.5057	1122.4910	0.0147	0	(32)	1,2	1	ASMTGGQQM
V	19	570.2505	1139.4964	1138.4859	0.0006	0	(39)	0.25	1	ASMTGGQQM
P	20	570.2553	1138.4961	1138.4859	0.0102	0	(39)	0.25	1	ASMTGGQQM
V	21	578.2548	1154.4950	1154.4808	0.0142	0	49	0.029	1	ASMTGGQQM
P	22	578.2559	1154.4972	1154.4808	0.0164	0	(34)	0.85	1	ASMTGGQQM
ঘ	23	578,2719	1154.5293	1154.4808	0.0486	0	(31)	1,8	1	ASMTGGQQM

Proteins ma	stching the	same set of	f peptides:		
gi 1084996	Mass	s: 2048	Score: 49	Queries matched:	6
Plasmepsin	II - malari	la parasite	(Plasmodium	falciparum)	
gi 1666274	Mass	s: 15059	Score: 49	Queries matched:	5
autoimmune	vaccine [sy	nthetic com	nstruct)		

# **Appendix A2:**

Result of MS/MS analysis of the  $\sim 17$  kDa band from SDS-PAGE.

Ions score is $-10*$ Log(P), where P is the probability that the observed match is a random event.						
Individual ions scores > 25 indicate peptides with significant homology. Individual ions scores > 28 indicate identity or extensive homology ( $p < 0.05$ )						
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.						
of Hits						
0 0 0 10 20 30 40 50 60 Probability Based Howse Score						
Peptide Summary Report						
FormatAs Peptide Summary Y						
Significance threshold p< 0.05 Max. number of hits 20						
Standard scoring ③ MudPIT scoring ○ Ions score cut-off 0 Show sub-sets □						
Show pop-ups 💿 Suppress pop-ups 🔿 Sort unassigned Decreasing Score 🛛 👱 Require bold red 🗌						
Select All Select None Search Selected Error tolerant						
1. 149669 Mass: 64628 Score: 52 Queries matched: 1						
FKBP65 binding protein - mouse						
Check to include this hit in error tolerant search						
Query Observed Mr(expt) Mr(calc) Delta Miss Score Expect Rank Peptide						
✓ 1 677.8360 1353.6574 1353.5983 0.0592 0 52 0.00024 1 K.TISDMFQHQDR.N						
Proteins matching the same set of peptides:						
ALL57621 Mass: 64656 Score: 52 Queries matched: 1 AF45641252 NTD: - Mus musculus						