STRUCTURE AND FUNCTION STUDIES OF FKBP65: A

PUTATIVE MOLECULAR CHAPERONE OF TROPOELASTIN

By

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ABSTRACT

FKBP-65 is a member of the immunophilin class of proteins consisting primarily of the cyclophilins and the FKBP's which bind the immunosuppressant drugs cyclosporin A and FK506, respectively. Immunophilins possess peptidylprolyl cis-trans isomerase (PPIase) activity which is inhibited upon binding of their respective macrolides. Specific cellular targets of most immunophilins and the role of PPIase activity *in vivo* remain largely unknown. FKBP-65 has been proposed as a molecular chaperone of tropoelastin (TE), the soluble precursor of elastin (Davis et al. 1998). TE contains 12% proline residues, many of which are found in VPGVG repeats. When P_2 is in the *trans* conformation, these motifs form repeated type-II β–turns and β–spirals resulting in self-association of TE *via* an inverse temperature-dependent transition known as coacervation. Coacervation can be monitored by turbidity increases at 300 nm. We have used purified recombinant FKBP-65 in coacervation assays with chick aorta TE to show that FKBP65 specifically affects the coacervation characteristics of TE in a concentration-dependant manner. The overall extent of coacervation of TE could be increased by more than 2-fold over controls by inclusion of nM amounts of FKBP-65 in the assay. Also, FKBP-65 decreases the coacervation onset temperature of TE by 5-10°C. Structural evidence suggests that the influence of FKBP65 on tropoelastin coacervation may be due to its ability to increase the β structural content of tropoelastin. These results suggest that FKBP-65 may be a physiologically relevant, TE-specific molecular chaperone.

ACKNOWLEDGEMENTS

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ABBREVIATIONS

ATP	- adenosine triphosphate
BiP	- immunoglobulin heavy chain binding protein
CBD	- chitin binding domain
CD	- circular dichroism
CsA	- cyclosporin A
C_T	- total coacervation
DTT	- dithiothreitol
EBP	- elastin binding protein
ER	- endoplasmic reticulum
FKBP	- FK506 binding protein
Hsp	- heat shock protein
IMPACT	- intein mediated purification with an affinity chitin-binding tag
IP_3R	- inositol 1,4,5-triphosphate receptor
IPTG	- isopropyl-α-D-thiogalactopyranoside
LB	- Luria-Bertani medium
pNa	- p-Nitroanilide
PCR	- polymerase chain reaction
PPIase	- peptidyl-proly-*cis-tans*-isomerase
SDS-PAGE	- sodium dodecyl sulfate polyacrylamide gel elctrophoresis
T_c	- coacervation temperature

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CHAPTER 1: INTRODUCTION

1.1 TROPOELASTIN AND ELASTIN

Elastic fibres are found in the extracellular matrix of elastic connective tissues and confer upon these tissues the characteristic of elasticity, that is, the ability to deform both repeatedly and reversibly upon application of mechanical stress (Vrhovski and Weiss 1998). Elastic fibres are primarily made up of two components: 1) an extensively cross-linked amorphous protein component known as elastin, and 2) a fibrillar component, the microfibrils (Ross and Bornstein 1969).

Elastin is the primary structural component of elastic fibres composing over 90% of its dry weight and, as such, is responsible for the main structural and functional features of the fibre. Elastin is an extremely insoluble protein being both highly cross-linked at Lys residues and amongst the most hydrophobic proteins known (Vrhovski and Weiss 1998). Elastin from higher vertebrates contains over 30% Gly and approximately 75% of the entire sequence is composed of only 4 hydrophobic residues, namely, Gly, Val, Ala and Pro. Various tissues rich in elastin include: aorta and major vascular vessels (28-32% dry mass), lung (3-7%), elastic ligament (50%), tendon (4%), and skin (2-3%) (Uitto 1979).

Various acquired and inherited diseases result from defects in the structure, distribution and abundance of elastin and elastic fibres thus underscoring the importance of studying elastic fibres, their components and other factors which may influence their synthesis, assembly and maintenance. Some of the more common diseases associated

with elastic fibre abnormalities include atherosclerosis, emphysema, supravalvular aortic

stenosis (SVAS), and Williams syndrome (Lowery et al. 1995).

TROPOELASTIN

1.1.1 Biosynthesis

Tropoelastin is the soluble, cellular precursor of elastin. Evidence suggests that

only one tropoelastin gene is present in the mammalian genome (Olliver et al. 1987). The

human gene has 34 exons and, as in many species, shows that hydrophobic and cross-

linking domains of tropoelastin are encoded by separate, alternating exons thus reflecting

the protein architecture (Bashir et al. 1989) (Figure 1.1.1).

Domain	Sequence
Hydrophobic Domain [Exon 24]	GLVPGVGVAPGVGVAPGVGVAPGVGLAPGVGVAPGVG VAPGVGVAPGIGPGGVA
Cross-link Domain [Exon 19]	GVVSPEAAAKAAAKAAKY
36A	GADEGVRRSLSPELREGGDPSSSQHLPSTPSSPR
C terminus	GGACLGKACGRKRK
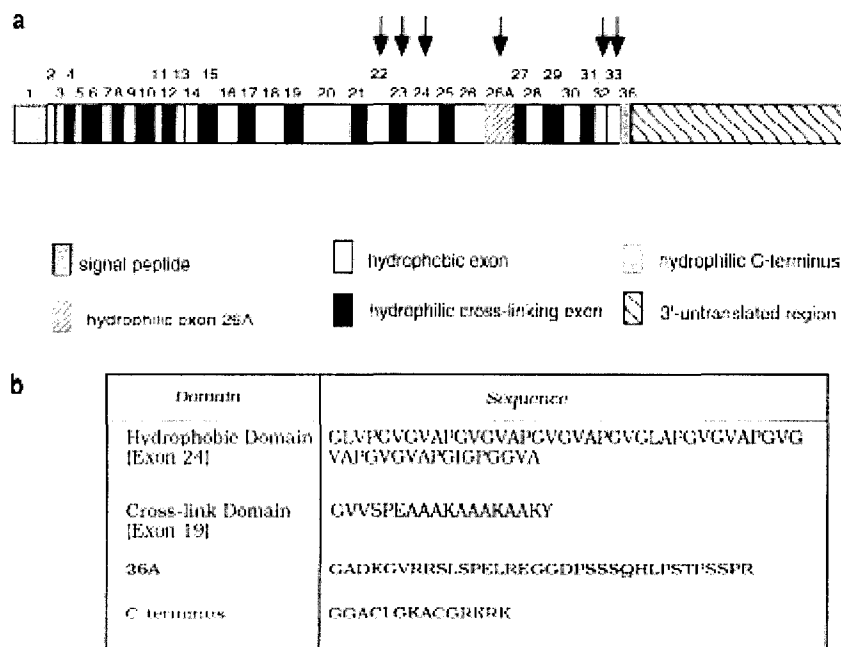
Figure 1.1.1. Structure of Human Tropoelastin. (a) cDNA structure. (b) Amino acid sequence of selected domains. Most of the gene consists of alternating hydrophobic and cross-linking domains. Exons subject to alternate splicing are marked with an arrow (Weiss, 1998).

Expression of tropoelastin mRNA is highest in early development and occurs within a limited time period (Parks et al. 1988). Tropoelastin expression is tightly regulated with a strong correlation between mRNA levels and tropoelastin synthesis. This suggests that expression levels are primarily controlled by pre-translational mechanisms (Burnett et al. 1982). Analysis of tropoelastin cDNAs has revealed significant variation in the nucleotide sequence within a species due to alternative splicing of tropoelastin mRNA (Baule and Foster 1988; Fazio et al. 1988a; Pierce et al. 1990). The result is that at least 11 human tropoelastin isoforms have been identified (Indik et al. 1987; Fazio et al. 1988a; Fazio et al. 1988b; Boyd et al. 1991). The role of different isoforms is currently unknown.

1.1.2 Secretion and Post Translational Modification

Translation of tropoelastin mRNA is targeted to the rough ER by a hydrophobic signal sequence (Saunders and Grant 1984). The molecule is then secreted into the lumen of the ER with release of the signal peptide where it may then interact with various ER localized molecular chaperones including BiP and FKBP65 (Davis et al. 1998). It has been shown that FKBP65 remains co-localized with tropoelastin as it traverses the ER and that the two proteins dissociate before reaching the Golgi apparatus (Patterson et al. 2000). The exact role of FKBP65 in tropoelastin biosynthesis remains unclear, however; a more detailed discussion of the possibilities will appear later in this thesis.

Tropoelastin undergoes little post-translational modifications with little evidence for gycosylation (Vrhovski and Weiss 1998). Hydroxylation of Pro residues by prolyl-hydroxylase varies from 0-20% (Uitto et al. 1976) but is believed to play no functional

role with overhydroxylation actually destabilizing the molecule (Urry et al. 1979). It is possible that Pro hydroxylation is merely a by-product of collagen Pro hydroxylation which occurs in the same cellular compartment at the same stage in development. Tropoelastin traverses the Golgi apparatus and appears to follow the classical secretory pathway through an acidic compartment to the cell surface where it is secreted into the extracellular space (Davis and Mecham 1996; 1998). Here, without any further modifications or proteolytic processing (Bressan and Prockop 1977), cross-linking occurs with an initial deamination of Lys residues by lysyl oxidase and the newly synthesized elastin molecule is incorporated into the growing elastic fibre.

The mechanism behind specific targeting of the tropoelastin molecule to the sites of fibre formation has recently been linked to the elastin binding protein (EBP) which binds both tropoelastin and laminin (Hinek et al. 1988). It is thought to localize tropoelastin at the cell surface where it can then interact with a galactosugar-or glycosaminoglycan-containing microfibril which results in the release of tropoelastin and the recycling of EBP back to the cell (Mecham et al. 1991). There is also evidence that EBP and tropoelastin form an intracellular complex and that EBP may function as a molecular chaperone protecting tropoelastin from degradation and self-aggregation (coacervation) (Hinek and Rabinovitch 1994).

1.1.3 Structure

The amino acid sequences of tropoelastins from various sources have been determined and all show significant homology both at the DNA and amino acid levels. Two major types of domains are present in tropoelastin: 1) hydrophobic domains rich in non polar amino acids like Glu, Ala, Val, and Pro and often existing in repeats of 3 to 6 residues such as VPGVG, GGVP, and GVGVAP and, 2) hydrophilic domains enriched in Lys and Ala which are involved in cross-linking and are usually in stretches of Lys separated by 2 or 3 Ala residues such as AAAKAAKAA (Figure 1.1.1) (Bashir et al. 1989).

The C-terminus of tropoelastin is highly conserved and very basic. It contains the only two Cys residues in the molecule and ends with the positively charged RKRK sequence. These two features of the C-terminus are strictly conserved and are believed to be essential for correct elastic fibre formation (Hinek and Rabinovitch 1993).

1.1.4 Coacervation

Tropoelastin has the remarkable ability to undergo a reversible inverse temperature transition known as coacervation. This is a phenomenon by which tropoelastin is soluble in cool aqueous solutions at less than $20^{O}C$; however, upon raising the temperature to the physiological range, ordered aggregation of the tropoelastin monomers occurs due to interactions between the hydrophobic repeat domains. Coacervation results in the onset of turbidity in the solution and can be monitored by following turbidity using a spectrophotometer. The process can be reversed upon cooling of the solution, however, if coacervated solutions are allowed to stand, a liquid-liquid phase separation occurs with the bottom layer forming a sticky, visco-elastic phase

containing highly concentrated tropoelastin and approximately 60% water (Urry 1988). The top layer is an aqueous equilibrium solution. The process of coacervation is thought to both concentrate tropoelastin and align cross-linking domains and is considered to be essential for fibrillogenesis (Urry 1978).

Remarkably, and as evidenced by CD studies, the process of coacervation seems to be an ordering process whereby tropoelastin molecules are converted from a state of very little order to one of significant structure (Urry et al. 1969). It has been shown that the VPGVG repeat of tropoelastin can form a type-II β turn with the Val[1] C-O oxygen hydrogen bonded to the Val[4] N-H hydrogen. Upon raising the temperature of such type-II β turn repeats in solution, the series of turns can wrap into a β spiral optimizing intramolecular hydrophobic contacts and allowing for intermolecular hydrophobic contacts to be made resulting in coacervation (Urry 1992). This process may explain the increase in order and, specifically, the increase in β structure observed when tropoelastin and other forms of experimental elastin coacervate (Urry et al. 1985).

1.2 FK506 BINDING PROTEINS (FKBPs)

1.2.1 Immunophilins

In 1984 Fischer et al identified an 18-kDa protein from porcine kidney which exhibited the novel activity of peptidyl prolyl cis-trans isomerization (PPIase) (Fischer et al. 1984). In the same year Handschumacher et al identified the primary binding protein of the potent immune inhibitor cyclosporin A (CsA) and named it cyclophilin (CyP) (Handschumacher et al. 1984). By 1989 it became clear that the two proteins were the same (Fischer et al. 1989). As well, in 1989 the cytosolic binding partner of another macrolide immunosuppressent FK506 (and its analog rapamycin) was identified (Harding et al. 1989). Interestingly, the protein also exhibited PPIase activity. However, it showed no homology to CyP. The protein was named FKBP for FK506-binding protein. Collectively, the family of proteins was termed immunophilins and their discovery led to an explosion of research into the PPIases resulting in the identification of over 30 CyPs and 20 FKBPs by 1998. Among the earlier discoveries was the fact that binding of CsA and FK506 by cyclophilin and FKBP, respectively, results in potent inhibition of the PPIase activity of these enzymes. However, PPIase inhibition is unrelated to the immunosuppressive function. Instead, immunosuppression results from a 'gain of function' mechanism involving the interference of immunophilin-drug complexes with various intracellular protein targets essential for T lymphocyte growth and differentiation (Schreiber 1991). This lead researchers to ponder the significance of PPIase function *in vivo*. Considering the widespread conservation of PPIases in all organisms from

archebacteria to primates, as well as the relatively stringent homology between active PPIase domains, it has been generally accepted that the immunophilins must play essential catalytic roles in many diverse cellular functions. However, detailed evidence for such functions has remained limited and the *in vivo* targets of all but a few immunophilins remain elusive.

1.2.2 FKBP12

FKBP12 was the original FKBP identified and contains the minimal FKBP like PPIase domain present in all the proteins contained within this sub-class of immunophilins. The main Ca^{++} storage compartment of mammalian cells is the endoplasmic reticulum. Release of stored Ca^{++} is primarily dependent on the activation of one of two receptors; the ryanodine receptors (RyRs) or the inositol 1,4,5-triphosphate receptors (IP$_3$Rs). FKBP12 has been shown to interact with both (Collins 1991; Cameron et al. 1995). The RyR consists of four individual 565 kDa subunits with 1 FKBP12 molecule per subunit (4 per tetramer). The heterocomplex dissociates with FK506 indicating that the interaction involves the active site of FKBP12. FKBP12 deficient receptors result in defective gating (Mayrleitner et al. 1994). However, substitution of FKBP12 with mutants showing drastically reduced PPIase activity result in normal Ca^{++} flux (Timerman et al. 1995). The results suggest that PPIase activity may not be essential for modulation of the calcium release channel.

More recent studies using mutant mice deficient in FKBP12 show severe cardiomyopathy and ventricular septal defects most likely attributed to altered single

channel properties of the cardiac RyR isoform RyR2 (Shou et al. 1998). These results strongly suggest that FKBP12 plays an important role in RyR function.

Similarly, FKBP12 was found to function in IP_3R channel activity. IP_3R is activated via phosphorylation by protein A kinase and is inactivated by calcineurin. Release of FKBP12 resulted in erratic channel activity and lead to the hypothesis that FKBP12 anchors calcineurin to IP_3R resulting in a more efficient negative feedback regulation of channel activity (Cameron et al. 1997).

FKBP12 has also been shown to interact with the transforming growth factor β type-1 receptor (TβR-1) (Huse et al. 1999) The interaction is dependent on the PPIase domain of FKBP12 and is believed to stabilize the receptor functioning as a negative regulator of TβR endocytosis. However, these claims remain controversial with some scientists asserting that the association between FKBP12 and TβR has no physiological importance. Considering the numerous interactions between FKBP12 and various other proteins, it is unsure what exact role is played by this FKBP and it is likely that it serves various tissue specific functions.

1.2.3 FKBP12 Structure/Function

Since FKBP12 was the first FKBP identified, it has been used to describe all of the structural and functional characteristics making up the common "FKBP domain". The structure of FKBP alone (Michnick et al. 1991; Moore et al. 1991) and in complex with FK506 (Van Duyne et al. 1991; Lepre et al. 1992; Van Duyne et al. 1993) has been solved both by X-ray crystallography and NMR spectroscopy. The human FKBP12

contains a five-stranded antiparallel β sheet wrapped in a right-handed twist around a

short α helix (Figure 1.2.1).
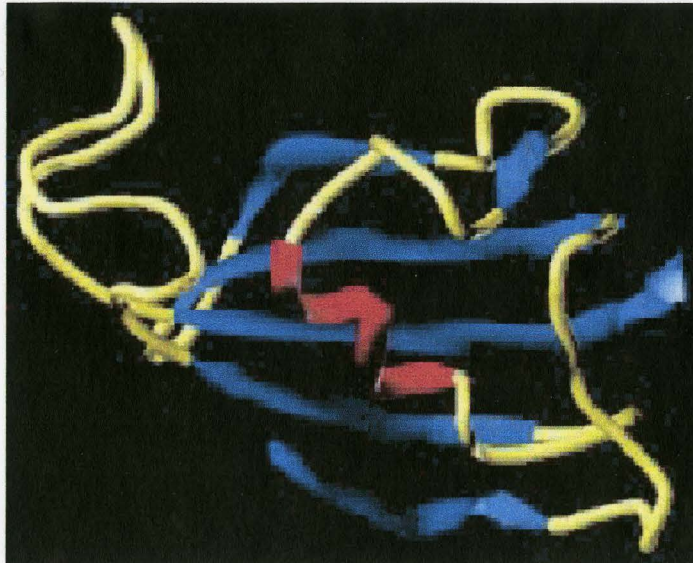
Figure 1.2.1. Ribbon diagram of FKBP12 structure. Figure illustrates five-stranded antiparallel β-sheet wrapped with a right handed twist around a short α-helix as well as the flexible connecting loops (Hamilton et al. 1998).

Various connecting loops can also be seen in the structure and are considered to

be quite disordered. The β structure is made up of residues 2-8, 21-31, and 35-38, and

twisting of the sheet results in both concave and convex hydrophobic surfaces. α

structure is comprised by residues 57-63 with residues 34-45 forming a loop connecting

the 2 parts of the third β strand (termed the 40's loop), and 84-91 forming a second loop

(the 80's loop). Various residues are strongly conserved with Tyr-26, Phe-36, Asp-37,

Val-55, Ile-56, Tyr-82, and Phe-99 being completely conserved in all known FKBPs with

significant PPIase activity (Hamilton and Steiner 1998). Interestingly, mutational analysis

has identified that PPIase activity and ligand binding can be structurally separated.

Mutating F36Y was found to more greatly affect PPIase activity rather than macrolide binding (Wiederrecht et al. 1992) as was the same with Y82L (Bossard et al. 1994). Mutating W59 to Ala resulted in a complete elimination of PPIase activity but not FK506 binding (DeCenzo et al. 1996). D37V (Aldape et al. 1992) or F99Y (Timerman et al. 1995) abolishes both PPIase activity and FK506 binding. These mutational results have been mirrored in the real world with divergent PPIase like sequences exhibiting intermediate functional properties to that of FKBP12. A good example of this is the case of FKBP52 (Chambraud et al. 1993) which is complexed with unliganded steroid hormone receptors. The protein has three FKBP like domains. The first N-terminal domain of FKBP52 has PPIase activity similar to that of FKBP12. Domain 2 shows increased divergence from the ideal PPIase sequence and has only 2% PPIase activity when compared to FKBP12. The third domain bears little resemblance to the classical FKBP12 domain and as might be expected, has no detectable PPIase activity. As well, Soldin et al. have isolated 37- and 52-kDa immunophilins which have both cyclophilin and FKBP domains. Both proteins have no detectable PPIase activity yet bind FK506, rapamycin, and CsA (Donnelly and Soldin 1994; Murthy et al. 1997). This leads to an interesting new proposition discussed in a thorough structural review by M. Ivery (Ivery 2000). In it, Ivery proposes that PPIase domains may be a new form of protein-protein interacting domain. Using extensive structural data of FKBP12 bound to various ligands, Ivery suggests that the enzyme selectively binds to peptides that can adopt a type VIa β-turn conformation. He goes on to say that ligand binding may serve to induce specific conformations in another region of the molecule effectively "switching" it on for protein

binding. Overall, Ivery suggests that PPIase activity itself may not be overly important, rather, specific structures resulting from prolyl bonds may act as conformation specific recognition sites.

1.3 FKBP65

FKBP65 was first discovered by Coss et al. from a murine JB6 epidermal cell cDNA library (Coss et al. 1995). It was identified due to a 46% overall homology with FKBP12. However, the FKBP65 proved to be somewhat different having a very unique protein architecture including a 33 amino acid N-terminal signal sequence, four PPIase-like domains, 2 EF-hand calcium binding motifs, and a C-terminal HEEL ER retention signal. The protein had a predicted mass of 64, 683 Daltons (thus FKBP65). However, after cleavage of the signal sequence, the mature protein was expected to have a mass of 60, 576 Daltons. The four PPIase like domains are located at residues 61-149, 173-261, 285-373, and 398-485 and represent 67% of the protein. The remaining regions flanking the PPIase domains seemed to be unique showing no homology to known sequences. At the time, Coss made no predictions as to the function of the newly discovered FKBP family member.

Several years later, Zeng et al. purified the same protein from chick embryo protein extracts (Zeng et al. 1998). Their interest was to investigate the PPIase influence on the refolding of type III collagen as prolyl isomerization had earlier been implicated as a rate limiting step in collagen folding (Bachinger et al. 1980). Zeng was able to show a small increase in the rate of type III collagen refolding in the presence of FKBP65. However, they concluded that a cyclophilin-type PPIase was more likely utilized by

collagen since adding CsA to collagen producing cells detrimentally affected collagen synthesis with FK506 having little effect (Bachinger et al. 1993).

Later in 1998 in an attempt to identify molecular chaperones of tropoelastin, Davis et al. identified a novel ligand for the immunophilin FKBP65 (Davis et al. 1998). Using bifunctional chemical cross-linkers and immuno-precipitation in bovine chondrocytes, Davis was able to identify two tropoelastin associating proteins, p78 and p74. Sequence analysis identified p78 and p74 to be BiP and FKBP65 respectively. Since tropoelastin is a molecule containing 12% proline residues, Davis hypothesized that FKBP65 may be involved in the isomerization of these residues and may ultimately play an important role in tropoelastin folding, trafficking and elastic fiber formation. A further discussion of these possibilities will follow.

1.3.1 FKBP65 Expression

Another unique aspect of FKBP65 is its expression pattern. Unlike most other FKBPs which are ubiquitously expressed, FKBP65 follows both a temporal and a tissue specific expression pattern. It is expressed in the testis, spleen, heart, brain, kidney, and is strongly expressed in vascular and airway tissues such as the lung (Coss et al. 1995; Patterson et al. 2000). It was also found to have expression limited to developing tissues with little or no expression seen in adult tissues (Patterson et al. 2000). Remarkably, the expression pattern observed for FKBP65 was very similar to that of tropoelastin thus increasing the likelihood that FKBP65 may be necessary for tropoelastin production. However, since FKBP65 is expressed in some non-elastogenic tissues, it is likely that more than one ligand exists for the protein.

1.3.2 Biochemical Characterization of FKBP65

FKBP65 is an acidic protein with a pI= 5.43. It has seven potential N-linked glycosylation sites and four possible phosphorylation sites. It is an identified glycoprotein and phosphoprotein (Coss et al. 1995). FKBP65 is an ER-resident protein as identified by immunofluorescence studies and co-localizes with tropoelastin in early secretory compartments with the two proteins dissociating before reaching the Golgi apparatus (Patterson et al. 2000). An earlier report by Coss et al. describing an association of FKBP65 with the cytosolic serine/threonine kinase c-Raf-1 (Coss et al. 1998) seems unlikely considering the fluorescence localization data.

Both Zeng et al. and Coss et al. have identified FKBP65 to be an active PPIase, albeit, with some discrepancies. Both groups used the α-chymotrypsin coupled assay for PPIase activity developed by Fischer (Fischer et al. 1984). The assay utilizes model substrates in the form Suc-Ala-Xaa-Pro-Phe-pNa where Xaa is any amino acid. In solution the X-Pro bond can exist in both the *cis* and the *trans* conformation. However, cleavage of the pNa bond by chymotrypsin is limited to those molecules with the X-Pro bond in the trans conformation. Once liberated, the pNa chromaphore can be monitored spectrophotometrically. An initial burst phase in the assay is due to the large percentage of X-Pro bonds (~90%) already existing in the trans conformation, however, the second slower phase of the assay is dependent on the *cis* to *trans* conversion of remaining molecules, a process which is accelerated in the presence of a PPIase. Using this method Zeng et al. identified that FKBP65 is an active PPIase with only one of the 4 PPIase domains being inhibitable by FK506 and, surprisingly, by CsA as well. No other FKBP

domain has ever been reported to bind CsA. Alternatively, Coss et al. reported that the

enzyme is completely inhibitable with FK506 and that CsA has no effect. The reason for

these discrepancies is puzzling and may be due to the high signal-to-noise ratio of the

coupled assay producing erroneous results.   It should be noted that out of the 4 PPIase

domains, domains I-III conserve 6 of the 7 essential residues for binding and catalysis

while domain IV conserves 5. It should also be noted that the residues corresponding to

W59 in domains I-III are changed to Met and in domain IV to Leu and that mutational

analysis of W59A produced an enzyme nearly incapable of PPIase function while still

being able to bind FK506 (DeCenzo et al. 1996).

1.3.3 FKBP65 Function

With the discovery in 1990 that the *cis* to *trans* conversion of peptidyl prolyl

bonds is a rate-limiting step in protein folding (Kiefhaber et al. 1990), it followed that

PPIases may function as molecular chaperones involved in directing the folding of newly

synthesized and mis-folded proteins. Considering tropoelastin contains 12% Pro residues

and has been shown to co-immunopercipitate with FKBP65, it seems likely that the

PPIase domains of FKBP65 serve some type of chaperone or foldase function. In

addition, many of the Pro residues in the tropoelastin molecule are in regions essential for

secondary structure formation. Of particular interest is a central region in which the

pentapeptide sequence VPGVG repeats 11 times. This region is hypothesized to form

repeated type II β-turns with the C-O of Val1 being hydrogen bonded to the N-H of Val4

(Urry et al. 1995). For this bond to form, Pro2 must be in the *trans* conformation. The

type II β-turns are considered essential in order to then form a stacked β-spiral believed

to align monomer hydrophobic domains and allow for tropoelastin self association/ organization, a process known as coacervation. A single *cis* Pro residue in this region could disrupt the formation of the β-spiral and lead to non-productive folding or aggregation. As well, these VPGVG repeats are capable of forming the type VIa β-turn considered by Ivery (Ivery, 2000) to be the ideal binding motif for FKBPs. Taken together, the evidence strongly suggests that FKBP65 may function as a tropoelastin specific molecular chaperone.

1.3.4 FKBP65 and Disease

Distinct similarities exist between the story of Hsp47, a collagen specific molecular chaperone, and that of FKBP65 and tropoelastin. The parallel regulation of Hsp47 and collagen in health is similar to that seen with tropoelastin and FKBP65. As well, Hsp47 and collagen expression are similarly disregulated in various fibrotic diseases of the liver, kidney, lung and dermis as well as in cancer (Van Duyne et al. 1991; Masuda et al. 1994; Razzaque and Taguchi 1997; Razzaque et al. 1998; Rocnik et al. 2000; Razzaque and Ahmed 2002). It follows that if FKBP65 can be shown to be a tropoelastin-specific molecular chaperone it may therefore be involved in various elastin related diseases. As such, FKBP65 could some day prove to be a feasible therapeutic target for vascular disease.

1.4 Objective of Thesis

The existence of molecular chaperones specific to tropoelastin seems likely but no definitive proteins have been identified to date. One attractive candidate is FKBP65 which has been found to co-localize with tropoelastin in the ER and immunoprecipitate with the protein. As well, the numerous proline residues of tropoelastin in regions essential for secondary structure may require *cis-trans* isomerization by FKBP65 or stabilization into specific type-II beta structures amenable to coacervation.

The objective of this thesis was to purify recombinant FKBP65 and use it to determine if it is indeed a molecular chaperone of tropoelastin. The purified, folded FKBP65 obtained in this study displayed very little PPIase activity, however, it did appear to influence the coacervation characteristics of tropoelastin as evidenced by both UV based turbidity assays and CD-based assays.

CHAPTER 2: MATERIALS AND METHODS

2.1 General Materials

General chemical reagents were obtained from either Sigma, BioShop, Gibco-BRL, BioRad, or Fisher Scientific. IMPACT™ cloning vectors, sequencing primers, chitin beads, and broad range protein markers were obtained from New England Biolabs. Chick aorta tropoelastin was purchased from the Elastin Products Company. Suc-Ala-Leu-Pro-Phe-pNa was obtained from Bachem.

2.2 Plasmids

pTYB4-FKBP65: pTYB4-FKBP65 was constructed by Christy Thomson, McMaster University, for overexpression and purification of FKBP65 from *E. Coli.* A pET21a-FKBP65 construct was received as a gift from Dr. Elaine Davis containing the full murine sequence of FKBP65. A cDNA encoding FKBP65 without the 33 amino acid signal peptide was PCR amplified. The vector was cleaved at Nco I and Sma I restriction sites with Sma I leaving a blunt end and Nco I leaving a cohesive end. The cohesive end was then filled in using DNA polymerase I, large (Klenow) fragment. T4 polynucleotide kinase was then used to add phosphate to the PCR product 5' hydroxyl terminus and a blunt end ligation was performed. Ligation reactions were then transformed into a supercompetent strain of *E. Coli* and screened for positive clones. Positive clones were then used to prepare DNA which was subsequently transformed into BL21(DE3*) *E.Coli.* The nucleotide sequence of the above plasmid was confirmed by sequence analysis

(MOBIX, McMaster University). The resulting construct added one extra Gly residue at the C-terminus of the FKBP65 target protein.

2.3 Commonly used Buffers

Lysis Buffer:	50 mM Tris pH 7.5, 200 mM NaCl, 0.05% Tween-20
Chitin Wash Buffer:	20 mM Tris pH 7.5, 50 mM NaCl, 0.05% Triton-X-100
Cleavage Buffer:	2 M Urea, 10 mM ATP, 500 mM $MgCl_2$ in Chitin Wash Buffer
Buffer A:	20 mM Tris pH 7.5, 100 mM NaCl
Buffer B:	20 mM Tris pH 7.5, 500 mM NaCl
PPIase Assay Buffer	40 mM HEPES pH 8.0
UV Coacervation Buffer	40 mM HEPES pH 7.5, 150 mM NaCl
CD Coacervation Buffer	20 mM Tris pH 7.5, 150 mM NaF

2.4 General Methods

SDS PAGE was performed using 7.5% Laemmli style gels unless otherwise noted. 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) with 10% (w/v) DTT. Gels were stained with coomassie blue (50% EtOH, 10% acetic acid, 0.1% coomassie blue) and destained with 10% acetic acid.

2.5 Large-scale overexpression and purification of FKBP65 from *E. Coli*

For the overexpression of FKBP65-Intein-CBD fusion protein, a 5 ml LB culture containing 100 µg/ml ampicillin (LB(AMP)) was inoculated from frozen cultures of pTYB4-FKBP65 containing BL21(DE3*) *E. Coli* and grown overnight (O/N) at $37^{O}C$ with shaking at 250 r.p.m. This 5 ml culture was then used to inoculate a 250 ml culture of LB(AMP) which was also grown O/N. The saturated 250 ml culture was inturn used to inoculate 20 L of LB(AMP) in a fermenter which was then grown at $37^{O}C$, 250 r.p.m, 15-20 L/min airflow until an OD_{600} of 0.6-0.8 was reached. At this point the temperature of the culture was reduced to $14^{O}C$ and expression of the fusion construct was induced by addition of IPTG to a final concentration of 1 mM. Growth was carried out O/N and cells were harvested by centrifugation at 6000 r.p.m. using 1 L centrifuge bottles. The resulting pellet (approximately 100 g) was resuspended in 200 ml of lysis buffer containing 4 complete protease inhibitor tablets. The cells were then lysed using a French Press at 16000 p.s.i. The lysate was then centrifuged at 12000 r.p.m for 20 minutes and the cleared, soluble lysate fraction was applied to approximately 300 ml of chitin beads equilibrated with lysis buffer. Mixing with chitin beads was carried out for 1 hour at $4^{O}C$. Loaded beads were then spun down using a table top centrifuge at approximately 700-1000 r.p.m. The supernatant was poured off and 2 batch washes were carried out using chitin wash buffer. The beads were then poured into a column and washed with 300 ml of 2 M Urea, 10 mM ATP, 500 mM $MgCl_2$ in chitin wash buffer in order to remove the majority of bound GroEL. 50 ml of 50 mM DTT in the above solution (cleavage buffer) was then applied to the column and allowed to flow until a strong odor was evident in the

exiting buffer. The chitin beads were then allowed to incubate in the 50 mM DTT solution for a minimum of 48 hours and a maximum of 72 hours at $4^{O}C$. Ten 3 ml fractions were then collected from the column and run on SDS-PAGE to identify protein containing fractions. Those containing protein were then combined and dialyzed into low salt buffer A (a minimum of 2 X 1 L rounds of dialysis with 1 L being left O/N). The protein sample was then loaded onto a 2.5 ml Q-Sepharose column equilibrated with buffer A, washed extensively with buffer A, then eluted with high salt buffer B. Finally, buffer exchange, removal of residual ATP and concentration of protein samples was achieved using ultrafiltration with concentrator membranes. This procedure yielded approximately 2.5 mg of pure protein. Smaller scale purifications using 4-8 L of culture were also performed yielding roughly proportional protein amounts.

2.6 Quantification of Purified Protein

The concentration of purified FKBP65 was determined either by the Bradford assay (BioRad) or by measuring the absorbance of the protein solution at 280 nm. The extinction coefficient of FKBP65 without the hydrophobic signal sequence was estimated to be 57855 cm^{-1} M^{-1} based on the number of tryptophans, tyrosines, and cysteines in the protein (Gill and von Hippel, 1989). Both the Bradford and OD_{280} methods gave identical results.

2.7 Peptidyl-prolyl-cis-trans-isomerase (PPIase) Assays

PPIase assays were performed in a coupled assay with chymotrypsin as described previously (Fischer et al. 1984; Standaert et al. 1990; Blecher et al. 1996). The substrate peptide used was N-succinyl-Ala-Leu-Pro-Phe-pNa. Assays were perfomed in a 1 ml

quartz cuvette and contained 500 μl peptide solution (100μ M) in assay buffer (40 mM HEPES, pH=8.0), plus 100 μl of assay buffer +/- enzyme. All assay reagents were used directly after storage on ice for a minimum of 10 minutes. The reaction was initiated by addition of 50 μl of 10 mg/ml chymotrypsin followed by rapid mixing of cuvette contents and immediate monitoring of absorbance changes at 390 nm for 90 seconds in a UV spectrometer at ambient temperature. Rapamycin was added to the assays as a 500 μM stock solution in high grade methanol. First order rate constants (k_{obs}) were calculated from the slope of the plot of the log of the difference between absorbance at steady state and absorbance at time t plotted against time (Harrison and Stein 1990a). Values for k_{cat}/K_m were calculated according to $k_{cat}/K_m = (k_{obs} - k_u)/[E]$ where k_u is the rate constant for the uncatalysed isomerization reaction and k_{obs} is the rate constant for the catalysed reaction in the presence of PPIase at a given concentration of [E] (Harrison and Stein 1990a).

2.8 Turbidity-Based Coacervation Assays

Chick tropoelastin assays were performed using a Cary 300 UV spectrometer equipped with a block temperature control attachment and software capable of raising the block temperature by $1^{O}C$ per minute from $15^{O}C$ to $65^{O}C$. Commercially purchased chick tropoelastin (Elastin Products Company) was solubilized in assay buffer (40 mM HEPES pH=7.5, 150 mM NaCl) to a concentration of 0.2 mg/ml. Individual assays contained 75 μl of a 0.2 mg/ml tropoelastin solution +/- the desired amount of protein in the same buffer. Assay volume was brought up to 300 μl using buffer. The final concentration of tropoelastin was approximately 700 nM. The solution was then placed in a 1 cm

pathlength quartz cuvette and placed into a UV spectrometer at $15^{O}C$ and allowed to equilibrate for 5 minutes. The temperature was then increased automatically by $1^{O}C$ per minute from $15^{O}C$ to $65^{O}C$ while monitoring turbidity at 300 nm. Rapamycin was added as a 500 μM stock in high-grade methanol.

Constant temperature assays were performed similar to above with some modifications. Firstly, more concentrated tropoelastin stock solutions were used in order to allow for greater volumes of FKBP65 to be added. Final volumes and tropoelastin concentration remained the same as stated above. Mixing of solutions was performed on ice then solutions were very quickly transferred to a 1 cm pathlength quartz cuvette and placed into a UV spec preheated to $45^{O}C$. Monitoring at 300 nm began immediately and proceeded for 30 minutes. Initial rates of coacervation were calculated from the slope of the plot of absorbance versus time between time points 1 minute to 3.5 minutes. Pre-equilibration of solutions to $45^{O}C$ before mixing was attempted by resulted in coacervation rates too rapid to monitor.

2.9 Structure Based CD Coacervation Assays

CD coacervation assays were performed using a Jasco J-600 spectropolarimeter attached to a Lauda thermostatically controlled re-circulating water bath. Chick aorta tropoelastin was solubilized to 0.1 mg/ml using 20 mM Tris pH=7.5, 150 mM NaF. Individual assays contained 270 μl of 0.1 mg/ml tropoelastin +/- enzyme in assay buffer. The final volume of the assay solution was brought up to 300 μl with buffer. The final concentration of tropoelastin was approximately 1.26 μM and was kept constant for all experiments. Spectra were obtained between 250-195 nm at 15, 25, 35, 45, and $55^{O}C$.

Our interest was in the structural changes of tropoelastin alone so as such it was necessary to obtain the appropriate spectra for subtraction before tropoelastin with FKBP65 could be monitored. This required that baseline spectra and FKBP65 spectra (at the concentration used with tropoelastin) be obtained at each temperature to be subtracted from later experiments with tropoelastin and FKBP65. All spectra were obtained using the same 0.2 cm jacketed quartz cuvette. Solutions were allowed to equilibrate to each temperature for 5 minutes before measurements were taken. Once collected, subtractions were performed using the Jasco software and data were converted to mean residue ellipticity. Data are displayed as comparisons between spectra of tropoelastin alone at each temperature and spectra of tropoelastin in the presence of FKBP65 at each temperature but with the contribution of FKBP65 at that temperature subtracted out.

Constant temperature assays were performed in the same manner but used more concentrated tropoelastin stock solutions to allow for greater volumes of FKBP65 to be added.

CHAPTER 3: RESULTS

3.1. Overexpression and purification of FKBP65 from *E. Coli*

3.1.1 Initial Protocol

The IMPACT system allows for the expression of one's target protein fused to the Intein-CBD construct. This allows for the purification of the target fusion protein from the soluble fraction of *E. Coli* lysates using chitin beads. Subsequently, one can obtain the target protein by inducing self-splicing of the intein element in the presence of thiols such as DTT. The Intein-CBD remains bound to the chitin column while the pure target protein should be eluted.

As aforementioned, pTYB4-FKBP65 was constructed by Christy Thomson at McMaster University. As well, a purification protocol was devised that resulted in purification of a protein which when run on SDS-PAGE appeared as a large band at approximately 61000 kDa with a small seemingly contaminant band slightly below the prominent one (Figure 3.1.1).
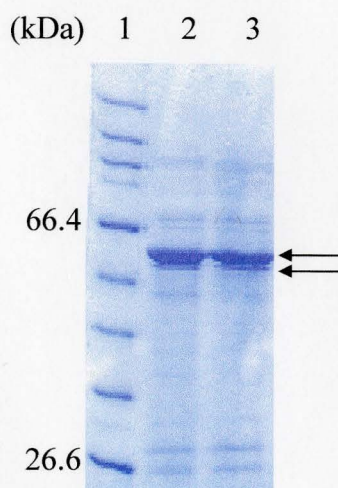
Figure 3.1.1. Coommassie Stained Gel of chitin elutions using initial procedure.
Elutions from a chitin column after incubation at 4^OC for 48 hours using the initial
purification procedure. Lane 1- MW markers, Lane 2- elution #1, Lane 3- elution #2.
Upper arrow indicates major protein component which was expected to be FKBP65.
Lower arrow illustrates what was thought to be a minor contaminant.

This initial procedure was modified slightly and used to obtain the protein. The

resulting protein was used in several variations of the chymotrypsin coupled PPIase assay

and appeared to be active. However, when reproducibility problems were encountered, a

closer examination of the PPIase assay used revealed that the data was misinterpreted.

This lead to a careful examination of different forms of the PPIase assay found in the

literature and to the adoption of a form found to be reliable and reproducible.

Unfortunately, using this assay, the protein did not appear to be active as a PPIase. As

well, other problems began to emerge. For example, there appeared to be significant

differences between the protein concentration determined by OD_{280} compared with the

Bradford protein determination assay. These findings led us to the conclusion that there

could be a problem with the protein and, as such, we sent two samples for amino acid

analysis, one to The Alberta Peptide Institute (API) at the University of Alberta and another to the Advanced Protein Technology Centre at the University of Toronto. Figure 3.1.2 illustrates the outcome of that analysis.
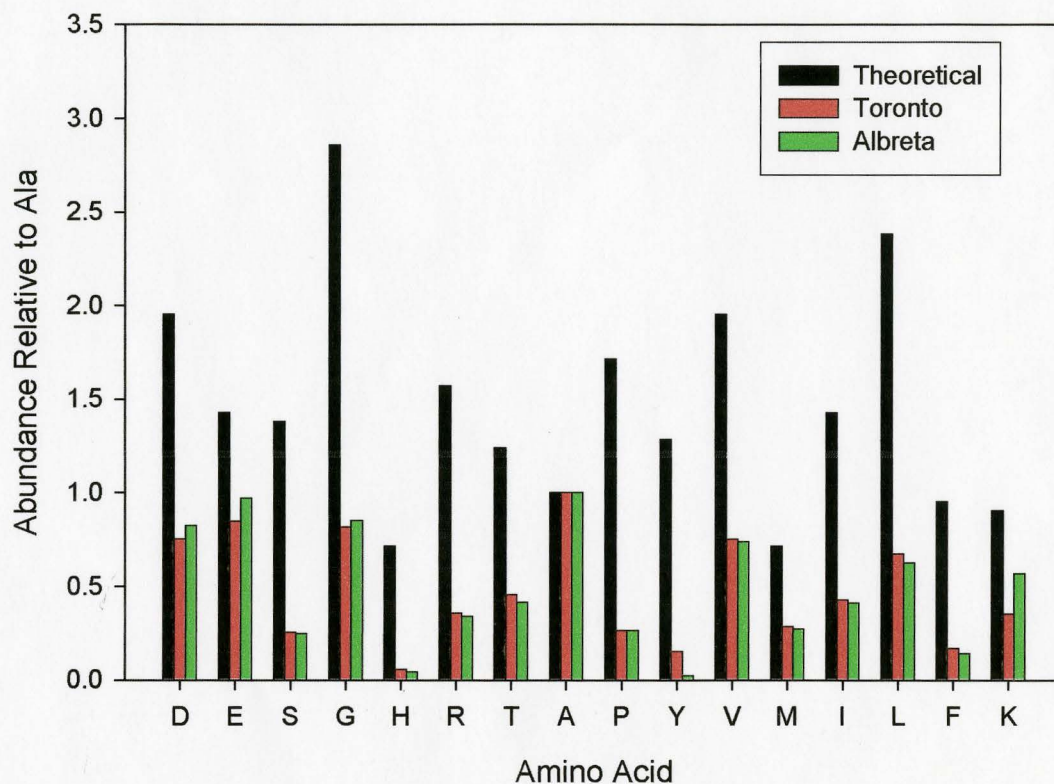
Figure 3.1.2: Amino Acid analysis results. Results from both The Alberta Peptide Institute (API) at the University of Alberta and the Advanced Protein Technology Centre at the University of Toronto are shown. Abundances relative to alanine are illustrated as ala tends to be a consistent amino acid with which to compare other amino acid values. Theoretical amino acid abundances were calculated from the cDNA sequence inserted into pTYB4.

Experimental amino acid composition values from each location were found to be consistent with eachother but drastically different from the theoretical composition of FKBP65. At this point it became apparent that the protein being purified, or at least the major protein component of the preparation, was in fact the wrong protein. Upon reexamination of the purification results, I began to suspect that the lower seemingly minor contaminant band which eluted with the larger band, may actually be the target FKBP65. I also noticed that older samples of the protein which had been stored at $4^{O}C$ did not seem to have the smaller band. To resolve the issue of what was FKBP65 (if any of the bands), two samples were run on SDS-PAGE, one with both bands and one with the one larger band. Both were then cut out of the gels and sent to the Institute for Biomolecular Design at the University of Alberta to undergo a process known as in gel digestion and protein identification. This is a process whereby the protein is trypsinized into peptide fragments which can then be analyzed by MS/MS spectrometry. The identity of the peptides can then be searched against a number of different databases (ie NCBI) and matches can be identified. Figure 3.1.3 illustrates the top 35 hits for the first sample which contained both bands.

```
Significant hits:

1. Chain A, Crystal Structure Of The Asymmetric Chaperonin Complex GroelGROES(ADP)7
2. 60 kDa chaperonin (Protein Cpn60) (groEL protein)
3. GroEL-like protein [Enterobacter aerogenes]
4. 60 kDa chaperonin (Protein Cpn60) (groEL protein)
5. 60 kDa chaperonin (Protein Cpn60) (groEL protein)
6. 60 kDa chaperonin (Protein Cpn60) (groEL protein)
7. 60 kDa chaperonin (Protein Cpn60) (groEL protein)
8. 60 kDa chaperonin (Protein Cpn60) (groEL protein)
9. 60 kDa chaperonin (Protein Cpn60) (groEL protein)
10.similar to GroEL protein [Klebsiella pneumoniae]
11.60 kDa chaperonin (Protein Cpn60) (groEL protein)
12.GroEL [Sitophilus oryzae principal endosymbiont]
13.GroEL protein [Candidatus Blochmannia floridanus]
14.60 kDa chaperonin (Protein Cpn60) (groEL protein) (Heat shock protein 60)
15.heat shock protein [Haemophilus influenzae Rd]
16.heat-shock protein GroEL - Pasteurella multocida
17.GroEL [Vibrio parahaemolyticus]
18.60 kDa chaperonin (Protein Cpn60) (groEL protein)
19.chaperonin, 60 Kd subunit [Vibrio cholerae O1 biovar eltor str. N16961]
20.GroEL protein [Buchnera sp.]
21.60 kDa chaperonin (Protein Cpn60) (groEL protein)
22.heat shock protein 60 kDa [Salmonella enterica subsp. enterica serovar Dublin]
23.60 kDa chaperonin (Protein Cpn60) (groEL protein)
24.Chain A, Crystal Structure Of The Molecular Chaperonin Groel Apical Domain
25.chaperonin GroEL [Shewanella oneidensis MR-1]
26.chaperonin hsp60 [Colwellia maris]
27.COG0459: Chaperonin GroEL (HSP60 family) [Azotobacter vinelandii]
28.GroEL [Erwinia amylovora]
29.60 kDa chaperonin (Protein Cpn60) (groEL protein)
30.60 kDa chaperonin (Protein Cpn60) (groEL protein) (63 kDa stress protein) (GSP63)
31.heat shock protein GroEL - Haemophilus ducreyi
32.60kDa chaperonin [Xanthomonas campestris pv. campestris str. ATCC 33913]
33.65kDa FK506-binding protein [Mus musculus]
34.chaperonin 60kD subunit [Chromobacterium violaceum ATCC 12472]
35.TCP-1 (Tailless complex polypeptide)/cpn60 chaparonin family
```

Figure 3.1.3. Results from in gel digestion and protein determination obtained from the Institute of Biomolecular Design, University of Alberta. As is evident, the greatest number of significant hits are for the *E. Coli* chaperonin GroEL. However, hit 33 is in fact FKBP65 with 9 identified peptides showing homology and the hit having an overall score of 257. Scores above 74 are considered to be significant.

At this point it became painfully apparent that FKBP65 was being bound by and co-purified with the E. Coli chaperonin GroEL and that GroEL was the primary component of the protein sample. A review of the literature revealed that this was not an uncommon problem and that it frequently could be solved by washing off GroEL with ATP and $MgCl_2$ solutions.

3.1.2 Revised Protocol

In light of this newly obtained information, many attempts were made to remove GroEL using various concentrations of ATP, $MgCl_2$, Urea, detergents and even inclusion of GroES (the chaperonin cap). Although much GroEL could be washed off, FKBP65 still eluted with GroEL as a major component. The most effective wash seemed to be 10mM ATP, 500mM $MgCl_2$, 2M Urea, all in chitin wash buffer. However, the cleavage step was always carried out in 50 mM DTT in chitin wash buffer as 2M Urea decreases the cleavage efficiency by 50%. This continued to result in co-purification of GroEL. Finally, the cleavage step was carried out in 10 mM ATP, 500 mM $MgCl_2$, 2 M Urea, in chitin wash buffer resulting in small amounts of pure FKBP65 being eluted. The identity of the eluted protein was confirmed by in gel digestion and protein identification which resulted in one significant hit, FKBP65.

Chitin fractions containing protein were then dialyzed into buffer A. In order to remove Triton-X-100 as well as smaller contaminant proteins, the protein was then applied to an equilibrated Q-Sepharose column, washed extensively, and eluted with high salt buffer B. Finally, buffer exchange, removal of trace ATP, and concentration of the

samples was accomplished using ultrafiltration. Figure 3.1.4 illustrates various steps of

the purification process.
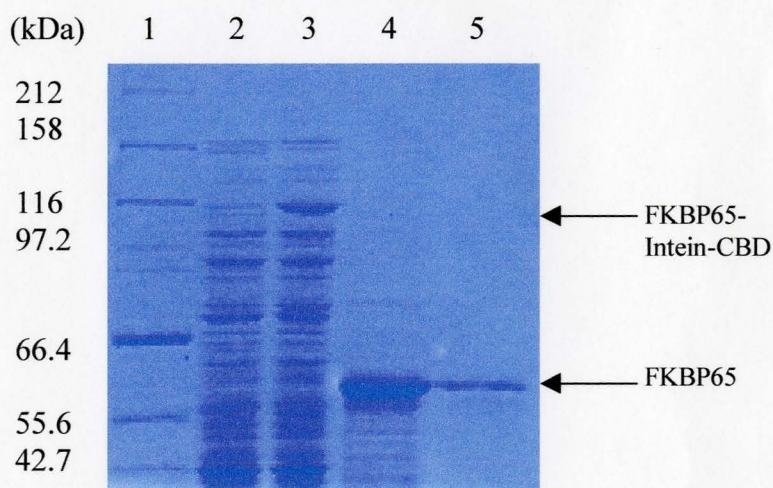
Figure 3.1.4. Purification of recombinant FKBP65 using the IMPACT^{TM} system.
Coomassie Blue stained SDS-PAGE gel illustrating various steps of the purification
process. Lane 1- broad range protein marker (NEB 7701S), Lane 2- Uninduced
BL21(DE3) cells, Lane 3- Induction of expression of the 116 kDa fusion protein with
1mM IPTG, Lane 4- Semi-pure FKBP65 eluted from a chitin column, Lane 5- Pure
FKBP65 eluted from a Q-sepharose column.

Unfortunately, the use of this procedure resulted in very poor yields of FKBP65.

Table 3.1.1 illustrates that out of 20 L of *E. Coli* culture, only 2.47 mg of pure FKBP65

could be obtained. Although inappropriate for initial hopes of crystal trials, the yield was

sufficient to carry out biochemical and structural characterization of the protein.

Table 3.1.1

Purification of FKBP65 from 20 L of Bacterial Culture

Fraction	Volume (ml)	Protein Concentration (mg/ml)	Total Protein (mg)
Cell Lysate	260	36	9360
Cleared Lysate	225	32	7200
Chitin	26	0.15	3.9
Q-Sepharose	9.5	0.26	2.47

3.1.3 Structural Analysis of Pure FKBP65

CD analysis of the purified protein as illustrated in figure 3.1.5 revealed a folded protein with over 60% beta structural content. This was encouraging as the secondary structure of FKBP12 is primarily beta with very little alpha structure. As well, purification of FKBP65 in our lab using a completely different construct and technique yields a similar protein structure. This leads us to believe that the structure obtained is that of native, folded FKBP65.
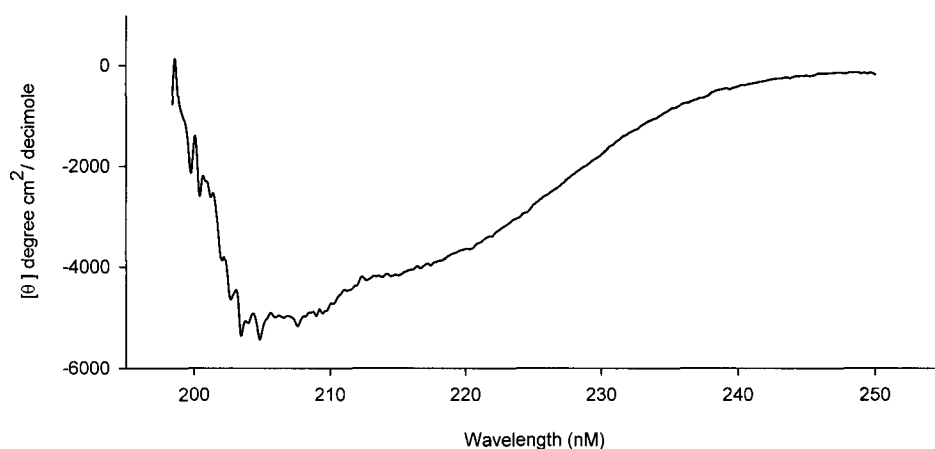
Figure 3.1.5. CD Spectrum of rFKBP65. Spectrum of purified FKBP65 in 20 mM Tris pH= 7.5, 150 mM NaF from 250 to 200 nm taken at 0.2 nm intervals. Secondary structural contributions were approximated to be 8.9% α-helix, 38.6% β-sheet, 21.1% β-turn and 31.1% other with and RMSD of 0.086 using the Contin method with 43 protein structures as a reference set.

3.2 Functional Analysis of FKBP65

3.2.1 PPIase Activity

As mentioned previously, FKBP65 has 4 PPIase like domains which each share varying homology with the traditional FKBP12 PPIase domain. Figure 3.2.1 shows the results of PPIase assays using both FKBP12 as a positive control and FKBP65. Figure 3.2.1 A shows the concentration-dependent activity of FKBP12 in accelerating the slow second phase of the chymotrypsin coupled reaction dependent on the *cis-trans* isomerization of the Leu-Pro peptide bond in the model substrate. B illustrates the concentration dependent inhibition of that activity by rapamycin. A and B illustrate the functionality and reproducibility of the assay performed as stated in the materials and methods. C illustrates the very low PPIase activity experienced with purified FKBP65 even when used in concentrations in excess of 10 times the concentration of FKBP12 needed to display significant activity. Also shown in C is the assay with the inclusion of 1.5 mM $CaCl_2$ as it was hypothesized that Ca^{++} ions may be necessary for activity due to the calcium binding motifs in FKBP65.

Table 3.2.1 and 3.2.2 illustrate the calculated rate constants from the progress curves of the previous figure as well as several of the published values for FKBP65 PPIase activity on 2 different substrates. As is shown, the PPIase activity of FKBP65 in our hands is negligible which conflicts with reports from other authors. Implications of this will be discussed later in this thesis.
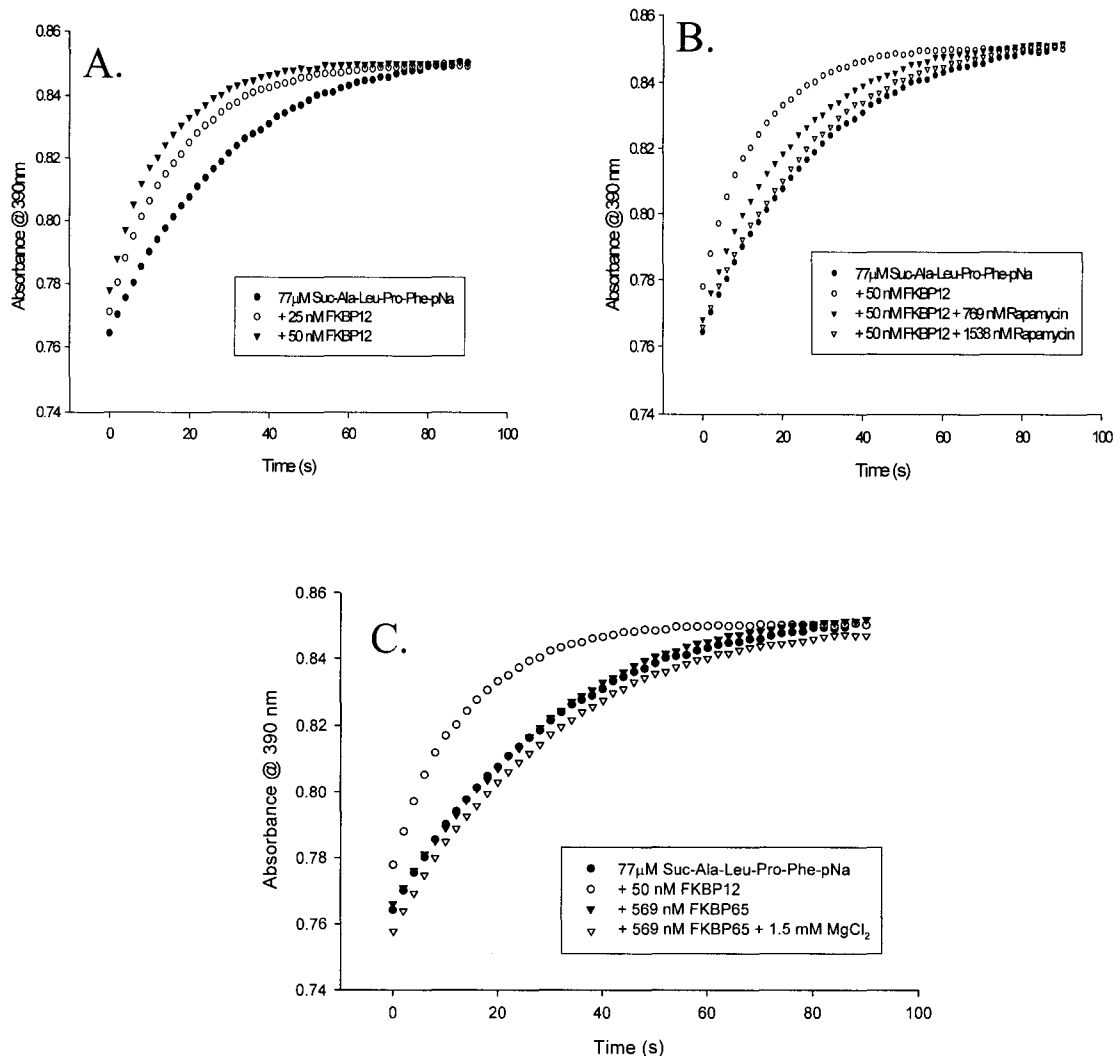
Figure 3.2.1. Peptidyl-prolyl-cis-trans-isomerase assays with FKBP12 and FKBP65.
(A) Kinetics of the hydrolysis of N-Suc-Ala-Leu-Pro-Phe-pNa without the presence of enzyme and with 25 and 50 μM FKBP12 illustrating concentration dependence of PPIase activity. (B) Partial and total inhibition of 50 μM FKBP12 activity with 769 nM and 1538 nM rapamycin respectively. (C) Kinetics of substrate hydrolysis in the absence and presence of 569 nM FKBP65. Assay also shown with inclusion of 1.5 mM $CaCl_2$. All assays performed in duplicate. Assays with FKBP65 repeated with three independent protein preparations and showed the same results.

Table 3.2.1 Kinetic Data for suc-Ala-Leu-Pro-Phe-pNa Substrate

Parameter		This Study ($t \sim 0^{\circ}C$)	Bachinger et al. ($t = 2^{\circ}C$)	Harrison et al. ($t = 10^{\circ}C$)
k_u ($10^{-3}s^{-1}$)		8.2 +/- 0.1	2.85	7.6 +/- 0.7
k_{obs} ($10^{-3}s^{-1}$)	FKBP12	14.75 +/- 2.6	52.0	---------
	FKBP65	8.7 +/- 0.2	16.3	---------
k_{cat}/K_m ($mM^{-1}s^{-1}$)*	FKBP12	131.0 +/- 52.3	330.0	640 +/- 48.0
	FKBP65	1.0 +/- 0.2	139.0	--------

Table 3.2.2 Kinetic Data for suc-Ala-Ala-Pro-Phe-pNa Substrate

Parameter		Coss et al. ($t \sim 20^{\circ}C$)	Bachinger et al. ($t = 2^{\circ}C$)	Harrison et al. ($t = 10^{\circ}C$)
k_u ($10^{-3}s^{-1}$)		---------	2.68	9.4 +/- 1.2
k_{obs} ($10^{-3}s^{-1}$)	FKBP12	---------	7.62	---------
	FKBP65	---------	16.3	---------
k_{cat}/K_m ($mM^{-1}s^{-1}$)*	FKBP12	---------	33.1	53.0 +/- 3.0
	FKBP65	640.0	80.3	--------

* Values for k_{cat}/K_m were calculated according to $k_{cat}/K_m = (k_{obs} - k_u)/ [E]$ where k_u is the rate constant for the uncatalysed isomerization reaction and k_{obs} is the rate constant for the catalysed reaction in the presence of PPIase at a given concentration of [E] (Harrison et al. 1990a).

3.2.2 Turbidity Based Coacervation Assays

As previously mentioned, one of the main goals of this project was to determine whether or not FKBP65 could function as a molecular chaperone to tropoelastin as had been suggested by the work of Davis et al. As such, we carried out experiments to determine the influence of FKBP65 on the self assembly process of tropoelastin known as coacervation. One way to monitor this process is by observing turbidity. With increasing temperature, tropoelastin is proposed to undergo a transition from relative disorder to a state of increased beta structure which allows for the alignment of hydrophobic domains between monomers resulting in self association and an increase in cloudiness or turbidity of tropoelastin solutions. Our hopes were that FKBP65 may be involved in the formation of the type-II beta spirals necessary for coacervation and therefore may influence the process. Excitingly, as shown in figure 3.2.2, FKBP65 was found to have a significant and concentration-dependent effect on the coacervation characteristics of chick aorta tropoelastin and was capable of more than doubling the total extent of coacervation. This novel effect was found to be specific to FKBP65 with both FKBP12 (shown) and BSA (not shown) at similar concentrations to that used with FKBP65, having little to no effect. Interestingly, the effect could not be inhibited with more than 8 times the molar concentration of rapamycin:FKBP65 leaving the role of PPIase activity in this process uncertain.
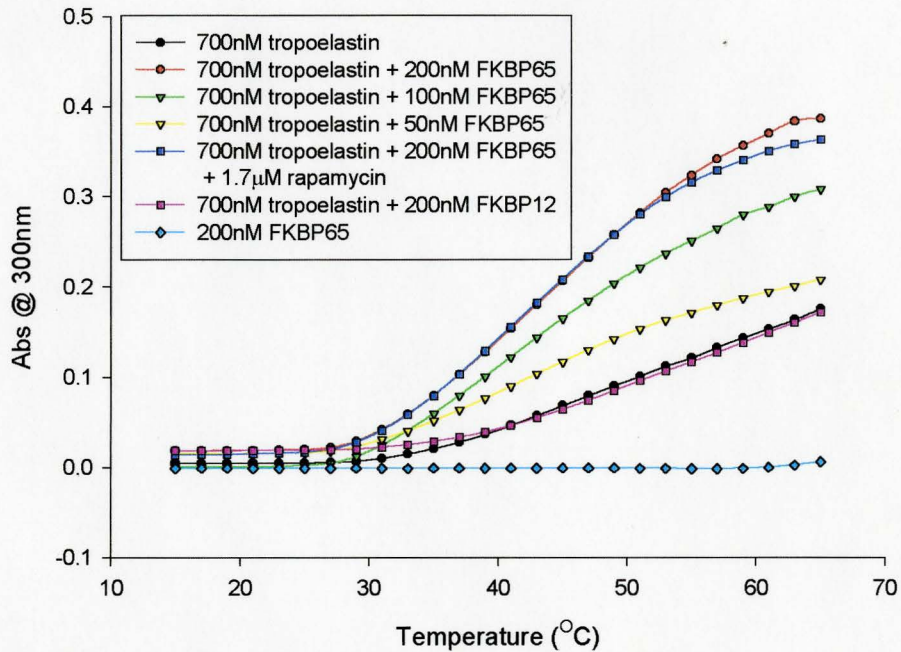
Figure 3.2.2. Effect of FKBP65 on coacervation characteristics of chick aorta tropoelastin. 300µl of 700nM chick aorta tropoelastin with and without varying concentrations of FKBP65 were placed in a UV spectrometer with $15^{O}C$ circulating water. The temperature was then increased $1^{O}C$/min up to $65^{O}C$ while monitoring turbidity at 300nm. Experiments were done in triplicate using 3 independent protein preparations. Representative data shown. FKBP12 negative control shown as well as inclusion of rapamycin. A run of FKBP65 alone is also shown.

Figure 3.2.3 illustrates the averaged results of triplicate data presented as mean values +/- standard deviation. As aforementioned, this figure illustrates the ability of 200 nM FKBP65 to more than double the overall extent of coacervation. The figure also illustrates the consistent concentration dependence of FKBP65 action on coacervation of chick aorta tropoelastin.
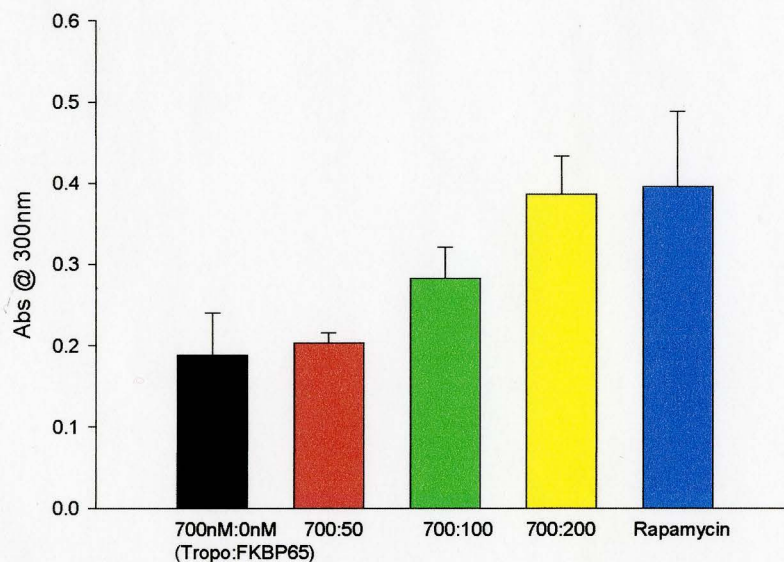
Figure 3.2.3. Effect of FKBP65 on total coacervation (C_t) of chick aorta tropoelastin. Summary of data in Figure 3.2.2 illustrating the effect of decreasing concentrations of FKBP65 on total coacervation (C_t) of 700nM tropoelastin. C_t is expressed as the final absorbance value at 300nm at 65^OC. Data presented as means +/- standard deviation, n=3. Rapamycin was included at a concentration of 1.7 μM in 200 nMFKBP65 runs.

Another interesting and informative aspect of tropoelastin coacervation is what is known as the coacervation temperature (T_c). Various factors can influence the point at which tropoelastin coacervates including the concentration of the protein, the salt concentration, and to a lesser extent pH. A number of different ways exist to evaluate T_c however, the one we have applied is illustrated in figure 3.2.4 and involves re-plotting the progress curves illustrated in figure 3.2.2 describing the turbidity changes as a percentage of the peak value at each FKBP65 concentration and taking the midpoint of these curves (ie the temperature at 50% turbidity) as the coacervation temperature. Interestingly, FKBP65 seems to again have a concentration-dependent effect resulting in lowered T_c with increasing FKBP65. This is evident as each % turbidity curve shifts to the left or to lower temperatures as FKBP65 concentration is increased. The result is a lowering of T_c by up to $10^{\circ}C$. Since lowered T_c reflects a greater propensity to coacervate, it seems likely that FKBP65 is behaving as a chaperone to tropoelastin assisting in secondary structure formation.
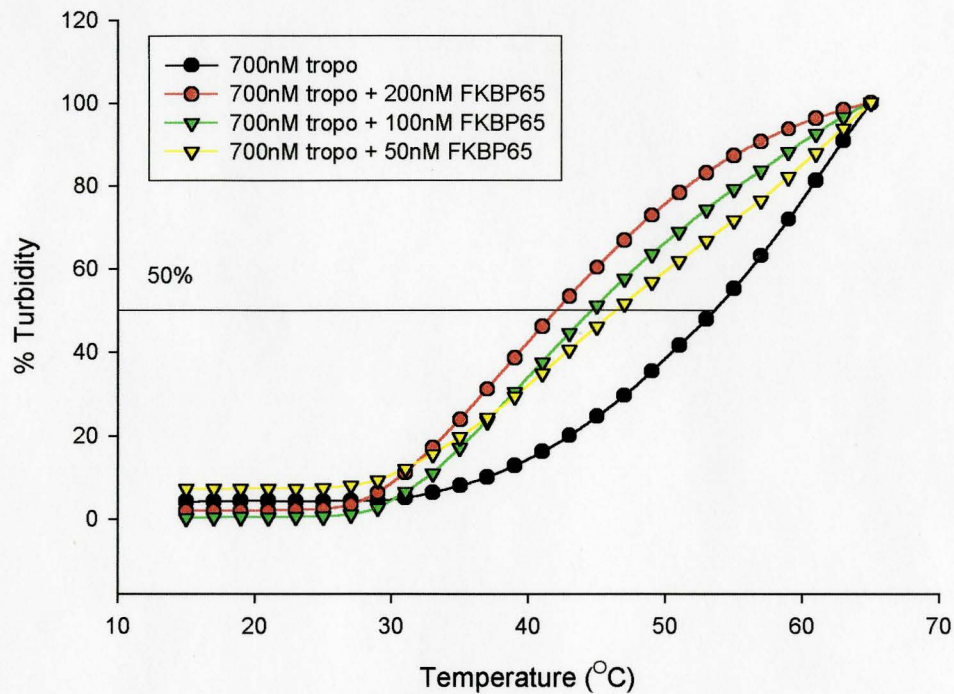
Figure 3.2.4. Effect of FKBP65 on coacervation temperature (T_c) of chick aorta tropoelastin . Coacervation curves derived from Figure 3.2.2 progress curves describing turbidity as a percentage of the peak value at each FKBP65 concentration. T_c is taken as the midpoint of these curves (ie. temperature at 50% turbidity). Experiments were done in triplicate using independent protein preparations. Representative data shown.

Figure 3.2.5 illustrates the averaged values of triplicate data on coacervation temperatures presented as means +/- standard deviation. This figure illustrates the concentration dependence of T_c lowering by FKBP65.
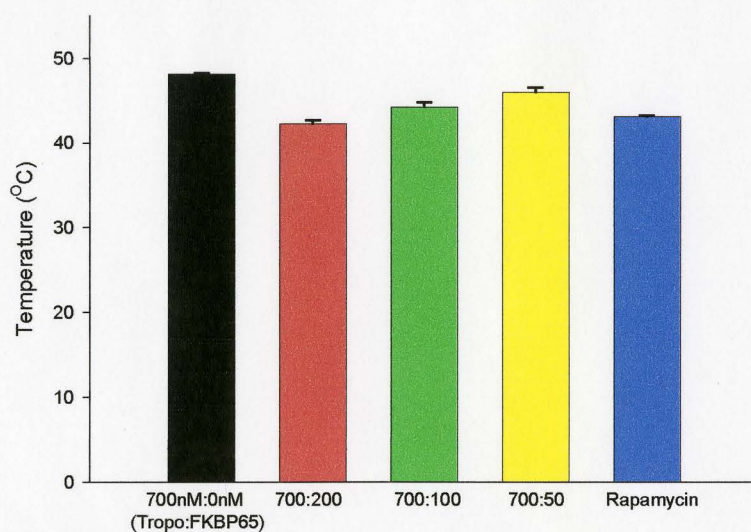
Figure 3.2.5. Effect of FKBP65 on coacervation temperature (T_c) of chick aorta tropoelastin . Summary of data in Figure 3.2.4 illustrating the effect of decreasing concentrations of FKBP65 on the coacervation temperature (T_c) of 700nM tropoelastin. T_c is expressed as the midpoint of the % turbidity curves illustrated in Figure 3.2.4. Data presented as means +/- standard deviation, n=3. Independent protein preparations used. Rapamycin was included at a concentration of 1.7 μM in 200 nMFKBP65 runs.

3.2.3 Structure-Based CD Assays

With the UV coacervation results established, we wanted to show that FKBP65 was actually increasing the beta structural content of tropoelastin and that this may in fact be the mechanism by which the observed influence on coacervation was occurring. To do this, we employed a CD based approach which offers much more information on the structural state of proteins. The goal was to examine the secondary structure of tropoelastin in the absence and presence of FKBP65 at varying temperatures. To do this, careful CD spectra of FKBP65 at the concentration used in the assay were first obtained for all the temperatures in question. These were then used to subtract the contribution of this protein from spectra in which both tropoelastin and FKBP65 were present thus leaving only spectral contributions from tropoelastin. Using this method we were able to compare the structure of tropoelastin alone at varying temperatures and the structure of tropoelastin in the presence of FKBP65 at each temperature. Figure 3.2.6 A illustrates the spectra obtained for 1.26 μM chick aorta tropoelastin at temperatures between 15-55°C. This type of pattern is typical of tropoelastin at increasing temperatures with a large minimum at approximately 200 nm which increases with higher temperatures. This rise is indicative of an increase in beta structure and is hypothesized to be the mechanism by which tropoelastin coacervates (Urry et al. 1985).
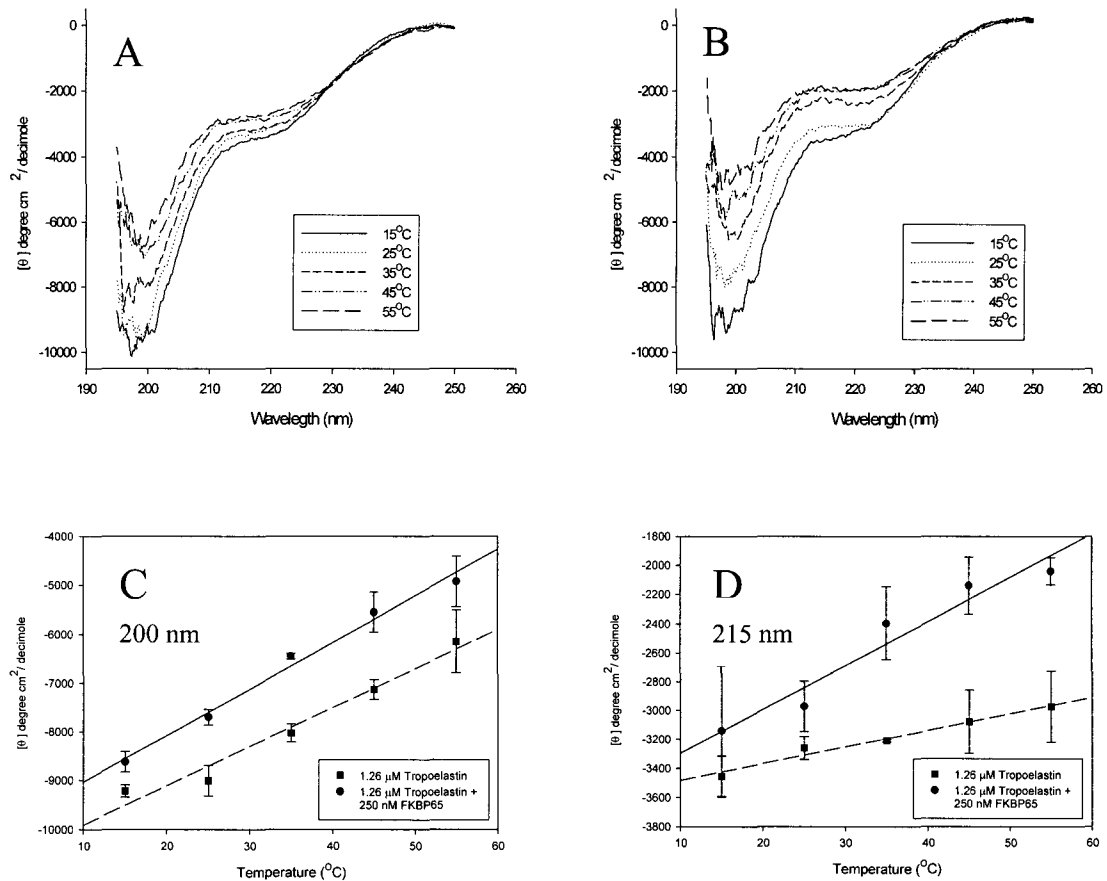
Figure 3.2.6. CD spectra of tropoelastin alone (A) and in the presence of FKBP65 (B) at varying temperatures. CD spectra of 1.26 μM chick aorta tropoelastin in 20 mM Tris pH=7.5, 150 mM NaF. (A) Spectra of tropoelastin alone obtained at varying temperatures after 5 minutes of incubation. Spectra are the averaged results of 8 runs. (B) CD spectra of tropoelastin in the presence 250 nM FKBP65 at varying temperatures after subtraction of FKBP65 structural contributions at each temperature. (C) Temperature dependent rise in tropoelastin ellipticity at 200 nm in the absence and presence of FKBP65. (D) Temperature dependent rise in tropoelastin ellipticity at 215 nm in the absence and presence of 250 nM FKBP65. Experiments performed in duplicate with independent protein preparations. Representative data shown.

Strikingly, and as illustrated in figure 3.2.7 B, the inclusion of 250 nM FKBP65 significantly increases the ellipticity at 200 and 215 nm at every temperature measured. This suggests that FKBP65 does increase the beta structure of tropoelastin and as such assists in β-spiral formation and coacervation of tropoelastin. The effect was found to be specific to FKBP65 with inclusion of BSA at almost 3 times the molar concentration of FKBP65 having little to no effect (Figure 3.2.7). C illustrates the temperature dependent rise in ellipticity at 200 nm while D shows the rise at 215 nm. Rises at these wavelengths are frequently used to assess coacervation. Both C and D illustrate the ellipticity rise with and without inclusion of 250 nM FKBP65. It is evident that the rise at each wavelength starts and ends at a higher value when FKBP65 is included and that the rise is steeper than that observed with tropoelastin alone (Figure 3.2.6 C and D). For instance, for one experimental run, the rise at 200 nm for tropoelastin alone is from -9131 deg cm^2/dmol at 15°C to -6601 deg cm^2/dmol at 55°C. The equivalent rise at 215 nm is from -3555 deg cm^2/decimole to -2798 deg cm^2/dmol. When FKBP65 is present, the rise at 200 nm is from -8773 deg cm^2/dmol at 15°C to -4561 deg cm^2/dmol at 55°C. That begins 358 deg cm^2/dmol higher than tropoelastin alone at 15°C and ends 2040 deg cm^2/dmol higher than tropoelastin at 55°C. This is considerably higher and suggests a considerable increase in beta structure. The rise at 215 nm is from -3459 deg cm^2/dmol at 15°C to -1974 deg cm^2/dmol at 55°C, also considerably higher than that with tropoelastin alone.

Figure 3.2.7 shows a comparison between tropoelastin structure with and without 250 nM FKBP65 at the physiological temperature of 35°C. As discussed above, the spectrum has significantly higher ellipticity at both 200 and 215 nm suggesting an increase in beta content. It is thus quite possible that FKBP65 behaves as a molecular chaperone of tropoelastin *in vivo* increasing its native like beta structural content and thus assisting in tropoelastin self association and elastic fibre formation.
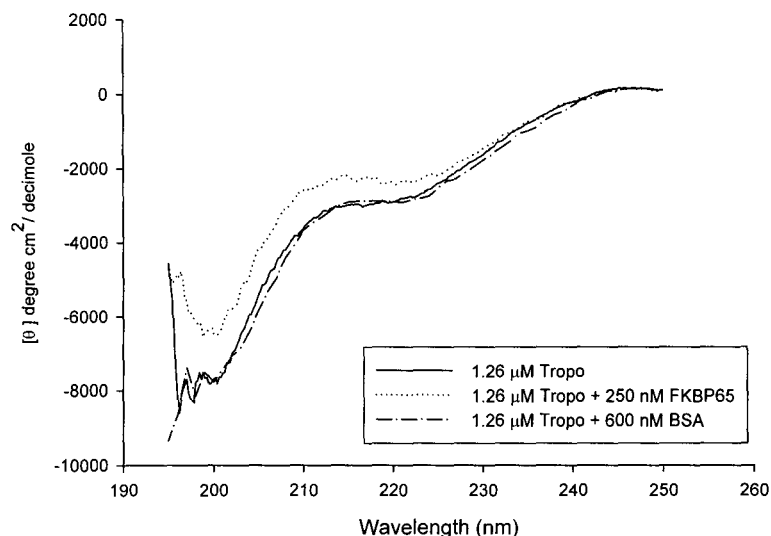
Figure 3.2.7. Comparison of CD spectra of tropoelastin at physiological temperature in the presence and absence of FKBP65 and BSA. Comparison of 1.26 μM tropoelastin at 35°C in the presence and absence of 250 nM FKBP65 and 600 nM BSA. Tropoelastin and tropoelastin + FKBP65 spectra taken from 35°C runs presented in Figure 3.2.6 A and B. Tropoelastin spectra with FKBP65 and BSA presented with structural contributions of FKBP65 and BSA subtracted.

3.2.4 Constant Temperature Coacervation Assays

In an effort to assess the stoichiometry of FKBP65 enhanced coacervation, we decided to employ a constant temperature turbidity based assay. By increasing the molar ratio of FKBP65:tropoelastin we hoped to reach a saturation point which might provide insight into the functional relationship between the two proteins. As illustrated in figure 3.2.8 A, the ability of FKBP65 to enhance tropoelastin coacervation seems to plateau at a 2:1 FKBP65:TE molar ratio. Interestingly, and as shown in B, the initial rate of coacervation also seems to plateau suggesting that FKBP65 may have maximum effects at these concentrations.
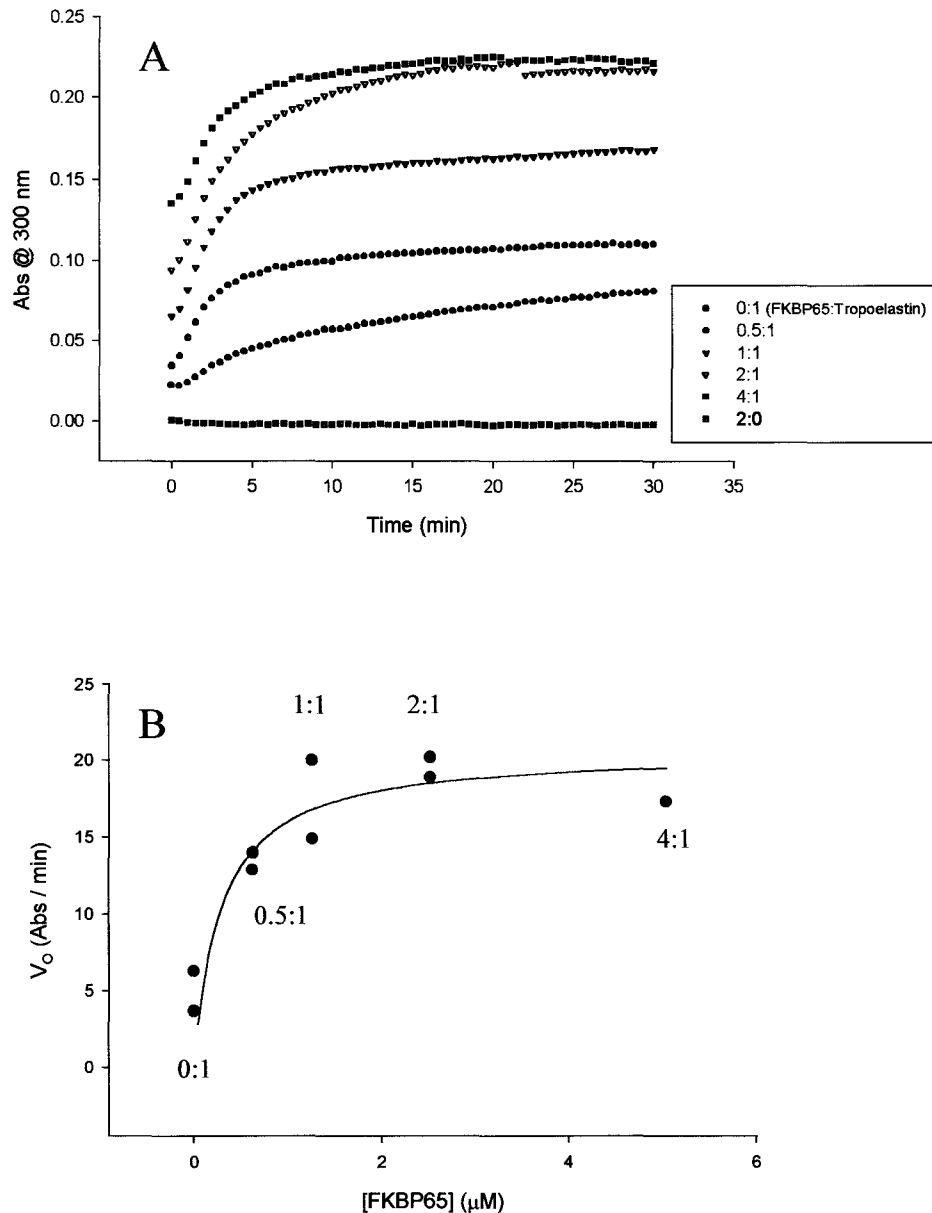
Figure 3.2.8. Constant Temperature Coacervation Assays. (A) 700 nM tropoelastin with FKBP65 at molar concentrations from 0:1 FKBP65:TE up to 4:1 was placed into a 1 cm quartz cuvette then into a UV spectrometer preheated to 45°C. Turbidity was monitored at 300 nm for 30 min (n=2). (B) Initial rates of coacervation plotted against FKBP65 concentration. Molar ratios of FKBP65:TE also included.

3.2.5 Constant Temperature Structure Based CD Assays

In order to check the results obtained in the constant temperature turbidity assay, we decided to take a similar approach with the CD based assay using a constant $45^{O}C$ and increasing the molar concentration of FKBP65:TE. The results are illustrated in figure 3.2.9 and show what seem to be maximum ellipticity increases at 200 and 215 nm at FKBP65 concentration of 2:1 or greater. However, the data obtained in these assays is difficult to interpret as higher concentrations of FKBP65 result in increased noise, especially below 200 nm. As well, a 4:1 FKBP65:TE experiment was unsuccessful as that concentration of FKBP65 results is extremely large structural contributions from FKBP which effectively drown out the tropoelastin signal and result in unfavorable noise levels even above 200 nm. As such, obtaining molar concentrations of FKBP65:TE greater than 2:1 in this assay is unattainable. However, 3.2.9 B does illustrate what may be a plateau in the elliptical rise at 215 nm at 2:1 FKBP65:TE on a ellipticity versus FKBP65 concentration plot.
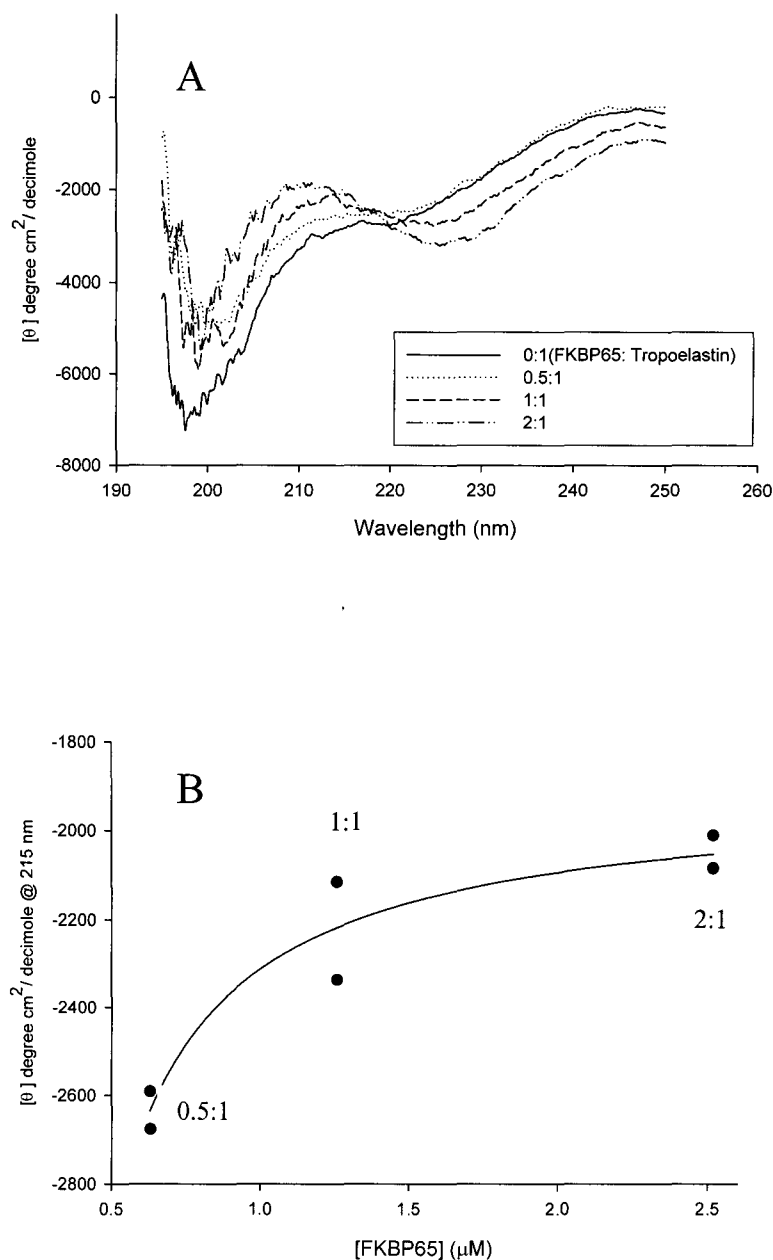
Figure 3.2.9. Constant Temperature Structure Based CD Assays. (A) 1.26 μM tropoelastin with FKBP65 at molar concentrations from 0:1 FKBP65:TE up to 2:1 was placed into a jacketed 0.2 cm quartz cuvette with recirculating water at 45°C. After 5 minutes equilibration spectra were gathered from 250-195 nm and averaged over 8 runs. Data are presented with structural contributions from FKBP65 at the applicable concentration subtracted (n=2). (B) Plot of the ellipticity at 215 nm versus FKBP65 concentration. Molar ratios of FKBP65:TE also included

CHAPTER 4: DISCUSSION

The IMPACT[TM] system for protein purification allows for relatively easy purification of a target protein with few purification steps. However, the fact that the proteins are purified form the soluble fraction of cell lysates increases the likelihood of co-purifying contaminant interacting proteins as occurred here with the co-purification of the *E. Coli* chaperonin GroEL. Solving this problem resulted in purification yields which were quite low (2.47 mg / 20 L culture) but sufficient for biochemical and functional characterization.

PPIase analysis of the purified FKBP65 revealed the protein to have very limited PPIase activity resulting in a mere 1.06 fold increase in k_{obs} over k_u even at concentrations in excess of 500 nM. This contrasts with the 5.7 fold increase seen by Bachinger (Bachinger et al, 1980) at 97 nM FKBP65 concentration using the suc-Ala-Leu-Pro-Phe-pNa substrate (see Table 3.2.1 and 3.2.2). Also contrasting are our k_{cat}/K_m values with Bachinger reporting 139 $mM^{-1}s^{-1}$ while we obtained a value of 1.0 +/- 0.2. As Bachinger purified FKBP65 from chick embryos, it is possible that post translational modifications play a role in the significantly larger PPIase activity seen by his group. However, it is also necessary to discuss the report of activity by Coss et al. Significant conflicts exist between the PPIase characterization of FKBP65 as published by Coss and Bachinger. The later reported that only 1/4 of FKBP65 PPIase activity could be inhibited by FK506 and could also be inhibited by CsA. This is the only report ever to show FKBP PPIase inhibition with CsA. Alternatively, Coss reported that FKBP65 could be

completely inhibited with FK506 and that CsA had no effect. Additionally, Bachinger reported a k_{cat}/K_m of 80.3 mM^{-1}s^{-1} for FKBP65 on suc-Ala-Ala-Pro-Phe-pNa. Coss reported the k_{cat}/K_m to be 640.0, a value more than 8 times that reported by Bachinger and more than 12 times larger than the k_{cat}/K_m reported by Harrison and Stein for FKBP12 (the most specific FKBP) on the same substrate (Harrison and Stein 1990b).

It is important to note here that Bachinger and Harrison's values for FKBP12 agree with each other. As can be seen in Table 3.2.1and 3.2.2, the k_{cat}/K_m values for FKBP12 on suc-Ala-Leu-Pro-Phe-pNa are 330 and 640 +/- 48.0 for Bachinger and Harrison respectively and 33.1 and 53.0 +/- 3.0 when suc-Ala-Ala-Pro-Phe-pNa is the substrate. The facts in this case lead to the conclusion that Bachingers activity seems to be reasonable while the Coss analysis is most likely a misinterpretation of the PPIase assay used.

Finally, as mentioned in the introduction, some important residues for PPIase activity are changed in FKBP65 PPIase domains. For example, the residues corresponding to W59 in domains I-III of FKBP65 are changed to Met and in domain IV, to Leu and mutational analysis of W59A produced an enzyme nearly incapable of PPIase function while still being able to bind FK506 (DeCenzo et al. 1996). This could result in an enzyme with PPIase activity so reduced that it is hard to detect. As well, a number of examples exist where proteins contain what look like PPIase domains however have no detectable activity. For example, Soldin et al. have isolated 37- and 52-kDa immunophilins which have both cyclophilin and FKBP domains. Both proteins have no detectable PPIase activity yet bind FK506, rapamycin, and CsA (Donnelly and Soldin

1994; Murthy et al. 1997). Also, in cases where more definite functional roles for FKBPs are known, as in FKBP12, elimination of the PPIase activity by mutation has no functional detriment (Timerman et al. 1995). So, it seems likely that an FKBP which exhibits little to no PPIase activity in the *in vito* assays may still be functional *in vivo*.

The real exciting revelation of this work has been the effect of FKBP65 on tropoelastin coacervation. Inclusion of FKBP65 in turbidity based coacervation assays results in more than doubling total coacervation and a reduction in the coacervation temperature by up to 10°C. This effect was shown to be specific to FKBP65 with BSA and FKBP12 having little to no effect. However, the mechanism by which FKBP65 enhances tropoelastin coacervation remains somewhat puzzling. Since FKBP12 had no effect, and rapamycin could not inhibit FKBP65's influence, the role of PPIase activity in the process of FKBP65 enhanced coacervation remains uncertain.

Some clues as to what may be happening are given by the CD structural data. Inclusion of FKBP65 with tropoelastin results in a significant upward shift in ellipticity at both 200 and 215 nm at all the temperatures studied (15-55°C). This upward shift is indicative of an increase in beta structure which has been shown to occur with coacervation in various elastin model systems (Urry et al. 1985). It is proposed that the increase in beta structure is necessary for aligning the hydrophobic domains of monomers of tropoelastin allowing for coacervation to occur. Thus, it seems likely that FKBP65 binds to tropoelastin resulting in an increase in beta structure in the protein. In this, FKBP65 acts as a chaperone to tropoelastin assisting in the folding of the protein into the native structure. Once again, the role of PPIase activity in this process remains uncertain.

However, when we look at the constant temperature saturation attempts (Figures 3.2.8 and 3.2.9) more clues to the overall process are given. In each of these experiments it seems that FKBP65's influence on tropoelastin coacervation saturates at molar ratios of 2:1 FKBP65:tropoelastin or greater. The fact that stoichiometric amounts are needed suggests that FKBP65 is acting more like a chaperone then a catalytic enzyme. As well, figure 3.2.8 illustrates a maximum absorbance at 330 nm of approximately 0.2 which is only attainable with 2:1 FKBP65:tropoelastin concentrations or greater. If the influence of FKBP65 on tropoelastin coacervation were dependent on successive catalytic rounds of *cis-trans* isomerization one would expect the process in figure 3.2.8 to continue on to 0.2 even for the lower concentrations of FKBP65 as more and more proline residues in tropoelastin are isomerized.

It seems that the data presented here supports Ivery's (Ivery, 2000) idea of the PPIase domain as a protein-protein interaction domain recognizing and possibly stabilizing specific type-II beta structures. It seems likely that FKBP65 binds to tropoelastin in the ER in stoichiometric amounts stabilizing native-like beta structure necessary for coacervation, then releasing tropoelastin at the Golgi network possibly by a decrease in pH associated with that cellular compartment. This idea is supported by fluorescence localization studies which show the association of FKBP65 and tropoelastin in the ER up to the Golgi network at which point they disassociate (Patterson et al. 2000).

In conclusion, this study has established that FKBP65 enhances tropoelastin coacervation. This effect is likely due to FKBP65's ability to increase the beta structure in tropoelastin. It is possible that FKBP65 binds to and stabilizes beta structure which is

essential for tropoelastin coacervation. However, it is also possible that efficient PPIase activity is necessary and intricately involved in this process. Future work will undoubtedly be necessary to determine what role, if any, is played by classical PPIase activity in tropoelastin coacervation. One initial approach underway in our lab is the cloning and purification of each of the four individual PPIase domains found in FKBP65 for kinetic analysis. Continued efforts at increasing our understanding of FKBP65 and other possible tropoelastin chaperones will almost certainly lead to an increase in understanding of elastic tissue in health and disease and could quite possibly lead to new methods to treat various elastic tissue disorders.

CHAPTER 5: REFERENCES

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