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Elastin Binding Protein and FKBP65 Modulate *in Vitro* Self-Assembly of Human Tropoelastin

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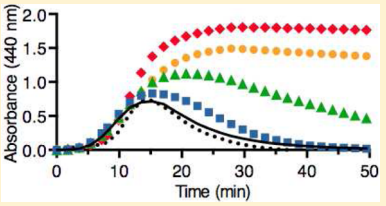
ABSTRACT: Elastin is a protein that provides the unusual properties of extensibility and elastic recoil to tissues. Assembly of polymeric elastin into its final architecture in the extracellular matrix involves both self-aggregation properties of its monomeric precursor, tropoelastin, and interactions with several matrix-associated proteins that appear to act by modulating the intrinsic self-assembly of tropoelastin. Because of its highly nonpolar character and propensity to self-aggregate, it has been suggested that mechanisms limiting self-aggregation must also be present during the transit of tropoelastin through the cell prior to secretion. Both the elastin binding protein (EBP) and FKBP65 have been suggested to fulfill that role in the Golgi and endoplasmic reticulum compartments of the cell, respectively. However, details about the nature of the interactions between these proteins as well as about the mechanism by which they may act to limit self-aggregation are lacking. In this study, we demonstrate that both EBP and FKBP65 have strong binding affinities for tropoelastin, with the dissociation constant of EBP approximately 4-fold lower than that of FKBP65. Both proteins also modify the kinetics of self-assembly of tropoelastin in an *in vitro* system, consistent with a role in attenuating the premature intracellular self-aggregation of tropoelastin through a mechanism that limits the growth and maturation of aggregates. The ability of FKBP65 to modulate the self-assembly of tropoelastin is independent of its enzymatic activity to promote the *cis*−*trans* isomerization of proline residues in proteins.



Elastin is the cross-linked, polymeric protein that provides the properties of extensibility and elastic recoil to tissues such as large arteries, lung, and skin. Assembly of the monomeric precursor, tropoelastin, into the extracellular elastic matrix is a complex process, requiring both the self-assembly properties of tropoelastin itself[1,2] and interactions with other matrix-associated proteins,[3,4] together determining the final architecture of the elastic matrix.

While significant progress has been made in the last several years in understanding interactions of tropoelastin with extracellular matrix-associated proteins, including fibrillins, fibulins, and MAGPs,[5−11] less is known about interactions between tropoelastin and other proteins during the intracellular transit of tropoelastin before secretion into the extracellular environment. This is an important consideration because tropoelastin is an unusually nonpolar protein, with more than 90% of its amino acids having uncharged side chains. Furthermore, computational modeling of the structure of elastin-like polypeptides (ELPs) has demonstrated dynamic, flexible conformations, which expose uncharged side chains to the polar environment.[12−15] Such conformational flexibility has been suggested to be essential for the elastomeric behavior of the elastin polymer[14,16] and likely accounts for the propensity of tropoelastin and ELPs to self-aggregate.

Self-aggregation of tropoelastin and tropoelastin-like polypeptides takes place through a process called coacervation, a rapid liquid−liquid phase separation resulting in the formation of protein-rich colloidal droplets.[2,17] *In vitro*, coacervation is usually induced by an increasing solution temperature, although the onset of aggregation can also be regulated by such factors as concentration and solution ionic strength.[2,18,19] Once considered to be only an *in vitro* phenomenon, coacervation of tropoelastin is now recognized as an important early step in the *in vivo* assembly of elastic fibres.[3,11,20−22] Indeed, several matrix-associated proteins have been shown to modulate the formation and maturation of colloidal droplets of coacervated tropoelastin and ELPs in both *in vivo* and *in vitro* systems.[8,11,17]

Given the propensity of tropoelastin to self-aggregate, it is reasonable to postulate mechanisms for the prevention or limitation of self-aggregation of tropoelastin during its intracellular transit prior to secretion. In particular, two proteins have been suggested to play such a role. The first of these, the elastin binding protein (EBP), was originally identified as a component of a cell-surface receptor for tropoelastin involved in the orderly addition of newly secreted protein to the growing elastic matrix.[23−25] Subsequently, this receptor has been shown to be a complex of EBP with neuraminidase-1 and cathepsin A and has been suggested to have a variety of signaling and other functions.[25−29] EBP itself has been reported to be a frame-shifted variant of β-galactosidase resulting in the loss of enzymatic activity and the gain of a binding site for tropoelastin.[30] Colocalization of EBP and tropoelastin within cells had prompted suggestions that EBP acts as a chaperone or companion protein for tropoelastin, preventing premature self-aggregation within the cell.[31,32]

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The second protein proposed to interact with tropoelastin during intracellular transport is FKBP65, based initially on reports of co-immunoprecipitation and intracellular colocalization[33] and subsequently supported by developmental and other studies.[34,35] More recently, FKBP65 had been reported to modify the self-assembly of tropoelastin in *in vitro* coacervation studies.[36] FKBP65 is a member of the immunophilin family of proteins and is a ligand for immunosuppressive drugs such as rapamycin and FK506.[37,38] FKBP65 contains four FK506 binding domains and a pattern of cysteines specific to endoplasmic reticulum (ER)-localized FKBPs.[39] FKBP65, like other members of this immunophilin family of proteins, has enzymatic activity for *cis*−*trans* isomerization of proline residues in proteins.[37,38,40] FKBP65 has also been demonstrated to exhibit chaperone activity in citrate synthase and rhodanase refolding assays[41] and has been reported to catalyze refolding of type III collagen[40] as well as to delay fibril formation of type I collagen.[41] More recently, loss of FKBP65 activity has been associated with forms of recessive osteogenesis imperfecta.[42−47]

Here, we investigate interactions between human tropoelastin and these two putative intracellular chaperones for tropoelastin, demonstrating that both EBP and FKBP65 bind to recombinantly expressed human tropoelastin and affect both the coacervation and maturation steps of the self-assembly process. In the case of FKBP65, these effects on tropoelastin self-assembly are independent of its peptidyl-prolyl isomerase activity.

EXPERIMENTAL PROCEDURES

Plasmid Construction. *Human Tropoelastin (hTE).* A full-length human tropoelastin cDNA (pUC19-hTE) was a gift from R. Mecham (Washington University, St. Louis, MO). The construct does not include exon 22 or 26A, neither of which is commonly expressed in cDNAs or present in the tropoelastin protein, and is identical to RefSeq NM_000501 except for the presence of the common G to S polymorphism in exon 20.[48] Briefly, the full-length hTE cDNA flanked by *Eco*RI sites from pUC19-hTE was inserted into pGEX-2T digested with *Eco*RI. To exclude the signal peptide (exon 1), an approximately 1 kb polymerase chain reaction (PCR) product was generated containing a *Bam*HI site, a start codon, and tropoelastin exons 2−18 (exon 18 contains a natural *Bam*HI site), using primers *Bam*HI-Ex2 and hTE1074-*Bam*HI with pUC19-hTE as the template. The 5′ portion (exons 1−18) of the original tropoelastin cDNA construct in pGEX-2T was excised by *Bam*HI digestion and replaced by the PCR product after *Bam*HI digestion, resulting in a human tropoelastin cDNA in pGEX-2T containing exons 2−36. The correct orientation and reading frame were confirmed by sequencing. Details of the construction of the hTE plasmid have been previously published.[49]

Human Elastin Binding Protein (EBP) and FKBP65. The cDNA clone for human EBP was a gift from A. Hinek (Research Institute, The Hospital for Sick Children). The cDNA clone for human FKBP65 was obtained from the PlasmID repository at the DF/HCC DNA Resource Core (Harvard Institute of Proteomics, Boston, MA).

For the EBP construct, a PCR fragment was generated containing the BM40 secretion signal sequence, the full-length human EBP cDNA, and a six-His repeat, using primer pairs EBP5′ and EBP3′, with EBP cDNA as the template. The PCR product was digested with *Hin*dIII and *Xho*I and ligated into a *Hin*dIII- and *Xho*I-digested pcDNA3.1+ vector to generate the pcDNA-EBP construct.

For the FKBP65 construct, a similar PCR fragment was generated containing the BM40 secretion signal sequence, the full-length human FKBP65 cDNA, and a six-His repeat, using primer pairs FKBP5′ and FKBP3′, with FKBP65 cDNA as the template. The PCR product was digested with restriction enzymes *Eco*RI and *Xho*I and ligated into an *Eco*RI- and *Xho*I-digested pcDNA3.1+ vector to generate the pcDNA-FKBP65 construct.

List of Primers: *Bam*HI-Ex2, TACGCGGATCCATGGGGGTCCCTGGGGCCATTC; hTE1074-*Bam*HI, ACCTGGGATCCCAGCACCTGGGA; EBP5′, CTTAAGCTTGCCTGCCTGCCTGCCACTGAGGGTTCCCAGCACCATGAGGGCCTGGATCTTCTTTCTCCTTTGCCTGGCCGGGAGGGCCTTGGCAGCCCCTCAGAGGATGTTTGAAATTGACTATAGC (this includes a *Hin*dIII site, a BM40 secretion sequence, and a 5′ EBP sequence); EBP3′, AGACTCGAGCTAGTGGTGATGGTGATGATGTACATGGTCCAGCCATGAATCTTTG (this includes a 3′ EBP sequence, a sequence for six His residues, and a *Xho*I site); FKBP5′, GTGGAATTCGCCTGCCTGCCTGCCACTGAGGGTTCCCAGCACCATGAGGGCCTGGATCTTCTTTCTCCTTTGCCTGGCCGGGAGGGCCTTGGCAGCCCCTGCCAGCCCGGCCGGGGGCCCCCTGGAA (this includes an *EcoRI* site, a BM40 secretion sequence and a 5′ FKBP65 sequence); FKBP3′, AGACTCGAGCTAGTGATGGTGATGATGGTGGACCCGCTCCTCGTCCTCATCTGA (this includes a 3′ FKBP65 sequence, a sequence for six His residues, and a *Xho*I site).

All oligonucleotide primers were synthesized by Integrated DNA Technologies (http://www.idtdna.com/), and construct sequences were confirmed using facilities provided by The Centre for Applied Genomics at The Hospital for Sick Children (http://www.tcag.ca/).

Recombinant Protein Expression. The procedure for expression and purification of human tropoelastin (hTE) followed previously described methods,[19] with some modifications. The hTE construct was transformed into BL21 cells, and a single colony was picked and grown in Terrific Broth containing 2% glucose. After induction with 0.1 M IPTG and further growth at 37 °C, the bacterial culture was harvested by centrifugation and digested with cyanogen bromide in 70% formic acid overnight at room temperature, followed by dialysis against water using a 25K cutoff dialysis membrane. The sample was then purified by reverse-phase high-performance liquid chromatography using a Jupiter C4 column.

Mammalian expression vectors pcDNA-EBP and pcDNA-FKBP65 were transfected into HEK293 cells using PolyFect transfection reagent (Qiagen). Stable lines were established using neomycin selection. Medium collected from the stable cell lines was concentrated by ultracentrifugation (Centricon Plus-70, Millipore) and buffer exchanged into 50 mM phosphate buffer (pH 8.0) containing 300 mM NaCl and 10 mM imidazole. The His-tagged recombinant proteins were then purified using Ni Sepharose beads (GE Life Sciences) and finally dialyzed into 50 mM phosphate buffer (pH 8) containing 300 mM NaCl. The molecular mass and purity of the recombinant proteins were confirmed using sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE), staining with PageBlue (Fermentas), and Western Blot analysis with an anti-human β-Gal antibody (Santa Cruz Biotechnology) and mouse anti-FKBP65 (BD Biosciences). Reduced samples were treated with 150 mM β-mercaptome-

thanol (150 mM) before electrophoresis. *N*-Glycosidase F treatment was conducted under denaturing conditions (0.1% SDS, 50 mM β-mercaptoethanol, and 5% Triton X-100) as recommended by the manufacturer (Calbiochem).

Circular Dichroism Spectrometry. Circular dichroism (CD) spectrometry experiments were conducted using a Jasco J-810 spectrometer. All CD experiments used a 1 mm pathlength quartz cuvette. Samples were dissolved in 50 mM phosphate buffer (pH 8.0) containing 300 mM NaCl. Spectra were acquired by stepwise scans using 1 nm increments and averaging the detected ellipticity for 4 s at each wavelength. Spectra shown in this study are the average of three independent scans. The spectra were deconvoluted using the K2D fitting algorithm on the Dichroweb server (http://dichroweb.cryst.bbk.ac.uk).[50] Thermal stability measurements were taken by heating samples at a rate of 1 °C/min and monitoring the average ellipticity at either 222 nm (EBP) or 217 nm (FKBP65) for each temperature increment.

Surface Plasmon Resonance (SPR) Interaction Analysis. Interactions between the EBP or FKBP65 protein and tropoelastin were investigated using the ProteOn XPR36 protein interaction array system (Bio-Rad). Tropoelastin in 10 mM acetate buffer (pH 5.2) was immobilized on a GLC chip (Bio-Rad) by an amine coupling method, following the manufacturer's instructions. All binding experiments were performed at 25 °C. Samples of EBP or FKBP65 were injected onto the chip at concentrations of 0, 1, 2, 4, and 5 μM for EBP and 0, 2.25, 9, 18, and 36 μM for FKBP65 in 50 mM phosphate buffer (pH 8.0) containing 300 mM NaCl. Injections onto the chip for association used a flow rate of 25 μL/min for 3 or 5 min. Dissociation was achieved by passing PBS buffer over the chip for 10 min at the same flow rate. Each binding interaction was performed at least twice, with five channels each time. After the blank had been subtracted, the curves were fit using the Langmuir binding model kinetic analysis with ProteOn Manager (Bio-Rad) to determine the association rate (k_a), the dissociation rate (k_d), and the dissociation constant ($K_D = k_d/k_a$) of the interactions. The χ^2 value, which describes the statistical measure of the closeness of the fit, showed good fits for the Langmuir model.

Coacervation. Coacervation experiments were conducted on a Shimadzu UV-2401PC UV–visible recording spectrophotometer with temperature and stir rate controllers, using 6.25 μM human tropoelastin in 50 mM Tris buffer (pH 7.5) containing 150 mM NaCl. Recombinant EBP or FKBP65 was included at molar ratios as indicated. The sample solutions were equilibrated at 25 °C for 5 min, and the temperature was then increased at a rate of 1 °C/min to 52 °C in the case of EBP and to 42 °C in the case of FKBP65, while the sample was being constantly stirred at 1000 rpm. Once the maximal temperatures had been reached, the solutions were held at these temperatures, while being stirred, for the remainder of the experiment. Parameters of *in vitro* tropoelastin self-assembly, including the temperature at which coacervation is initiated and the velocities of the coacervation and maturation steps, were determined using methods described previously,[17] based on at least three independent experiments. EBP was denatured by being boiled for 5 min in 0.1% SDS and 50 mM β-mercaptomethanol. Inhibitors of PPI activity were added to FKBP65 solutions before tropoelastin was added. Effects of galactose-containing carbohydrates on coacervation were determined using 30 mM lactose or 200 μg/mL chondroitin sulfate A.

Peptidyl-prolyl Isomerase Activity. The peptidyl-prolyl isomerase activity of recombinant human FKBP65 was measured as described by Harrison and Stein,[51] with minor modifications. The substrate, *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (Sigma Chemicals), was dissolved to a concentration of 100 μM in 50 mM Tris buffer (pH 7.4) and mixed with recombinant FKBP65 at a series of concentrations as indicated. α-Chymotrypsin (Sigma Chemicals) was then added to the reaction mixture to a final concentration of 12 μM, and the absorbance at 395 nm was immediately monitored for 100 s using a Shimadzu UV-2401PC UV–visible recording spectrophotometer. The observed rate constant for each enzymatic reaction was derived by least-squares fitting of absorbance versus time plots to a single-exponential rise-to-max function. K_{cat}/K_m was determined by plotting the first-order rate constant (k_{obs}) versus the FKBP65 concentration. Human FKBP12 (FK-binding protein, Sigma Chemicals) was used as positive control for isomerase activity. FK506 (Cell Signaling Technologies) at 10 μM and rapamycin (Sigma Chemicals) at 10 μM were used as inhibitors in the assay.

Light Microscopy. The onset of solution turbidity and maturation of the coacervate were imaged in real time using an Axiovert 200 inverted epifluorescence microscope (Zeiss). In each case, tropoelastin (25 μM) was prepared in coacervation buffer [50 mM Tris (pH 7.4) and 150 mM NaCl]. FKBP65 or EBP was added as indicated to a final concentration of 2.5 μM. Samples were transferred to an Attofluor cell chamber mounted in a temperature-controlled stage held constant at 45 °C. Bright field images were taken 60 min after the onset of solution turbidity, and droplets were analyzed using Volocity version 6.1.1.

RESULTS

Recombinant Human EBP and FKBP65. SDS–PAGE analyses of recombinant human EBP and FKBP65, expressed in the mammalian cell system and purified as described above, are shown in Figure 1. In the case of EBP (Figure 1A), PageBlue staining showed a major band at approximately 60 kDa (lane 2,

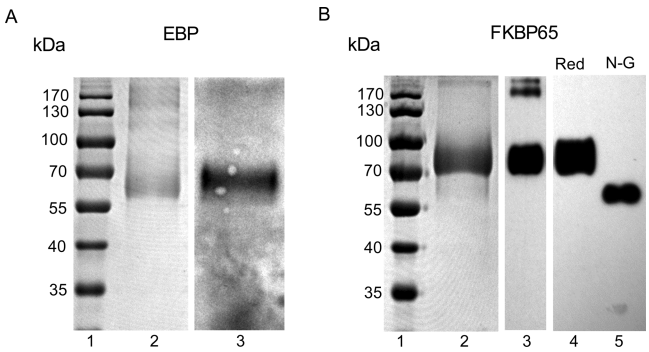

Figure 1. SGS–PAGE analyses of recombinant human EBP and FKBP65. (A) Purified recombinant human EBP visualized with PageBlue (lane 2) and by Western blotting (lane 3) with a polyclonal anti-human β-Gal antibody. The expected molecular mass is 58487 Da. (B) Purified recombinant human FKBP65 visualized with PageBlue (lane 2) and by Western blotting (lanes 3–5) with the monoclonal anti-mouse FKBP65 antibody. Abbreviations: Red, reducing conditions (150 mM β-mercaptoethanol); N-G, *N*-glycosidase F treatment under denaturing conditions (see Experimental Procedures). The expected nonglycosylated molecular mass is 61225 Da. Lane 1 in both panels shows molecular mass standards for SDS–PAGE.

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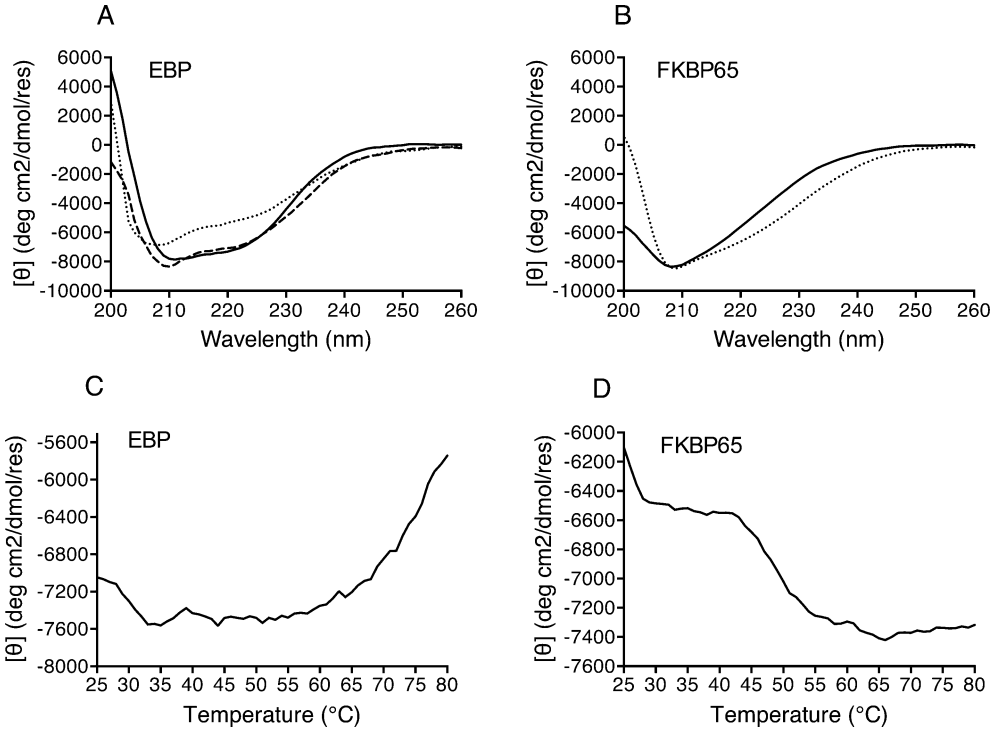

Figure 2. Secondary structure of recombinant human EBP and FKBP65. (A) Circular dichroism spectra of EBP at 25 (—), 70 (– – –), and 80 °C (···). (B) Circular dichroism spectra of FKBP65 at 25 (—) and 90 °C (···). Spectra were recorded in 50 mM phosphate buffer (pH 8.0) containing 300 mM NaCl. (C and D) Thermal denaturation of recombinant EBP, monitored at 222 nm, and FKBP65, monitored at 217 nm, respectively.

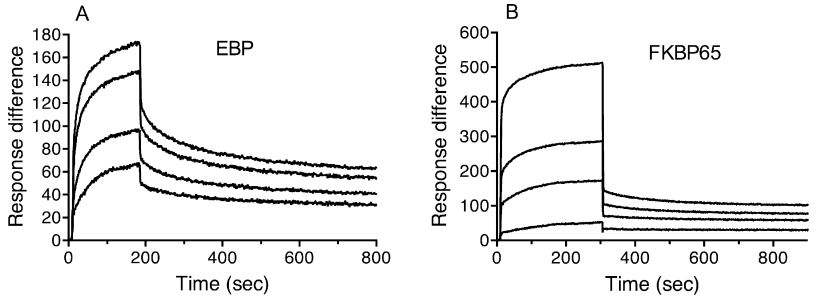

Figure 3. Molecular interactions of recombinant human tropoelastin (hTE) with EBP and FKBP65. (A) Surface plasmon resonance analysis of interactions between tropoelastin and EBP at concentrations of 1, 2, 4, and 5 μM (from bottom to top, respectively). (B) Surface plasmon resonance analysis of interactions between tropoelastin and FKBP65 at concentrations of 2.25, 9, 18, and 36 μM (lower to upper lines). Control sensorgrams (0 μM EBP or 0 μM FKBP65, respectively) were subtracted. One representative sensorgram for each pair of proteins is shown.

expected molecular mass of 58487 Da), which was also identified by Western blotting using a polyclonal antibody to human β-galactosidase (lane 3). In the case of FKBP65 (Figure 1B), PageBlue staining showed a predominant band at approximately 75 kDa (lane 2). Western blotting using a monoclonal antibody to mouse FKBP65 demonstrated a major reactive band at approximately 75 kDa as well as two additional bands of much higher molecular mass, both of which disappeared on electrophoresis under reducing conditions (lane 4), suggesting the presence of multimers formed through disulfide bridges. *N*-Glycosidase F treatment of the recombinant human FKBP65 (lane 5) reduced the mass to approximately 60 kDa, confirming that the protein was N-glycosylated by the expression system,[37] and consistent with a predicted nonglycosylated molecular mass of 61225 Da.

Secondary Structure and Thermal Stability of Recombinant EBP and FKBP65. The conformational states of EBP and FKBP65 were investigated using far-UV circular dichroism (CD) spectrometry (Figure 2). Deconvolution of the spectrum of EBP (Figure 2A) indicated 32% α-helix, 18% β-sheet, and 50% random coil, while that of FKBP65 (Figure 2B) showed 15% α-helix, 32% β-sheet, and 53% random coil. The latter values are in agreement with the proportions of these secondary structure elements previously determined by CD measurements for chicken FKBP65[41] and are consistent with five antiparallel β-strands and a short α-helix reported for the crystal structure of the homologous domain of FKBP12, which is repeated four times in FKBP65.[52]

Conformational stabilities of EBP and FKBP65 were determined by thermal denaturation, monitoring at wavelengths of 222 and 217 nm, respectively. EBP is highly stable, showing no structural melting until approximately 65 °C (Figure 2C). In contrast, FKBP65 is significantly less thermally stable, with loss of structure beginning at approximately 45 °C (Figure 2D).

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Table 1. Kinetic Analysis of Interactions of EBP and FKBP65 with hTE (6.25 μM) [mean ± SD (n = 5)][a]

	k_a ($\times 10^3$ M^{-1} s^{-1})	k_d ($\times 10^{-3}$ s^{-1})	K_D (nM)	χ^2
hTE−EBP	9.38 ± 0.57	0.76 ± 0.01	80.9 ± 4.5	11.1 ± 1.6
hTE−FKBP65	1.21 ± 0.06	0.44 ± 0.02	361.3 ± 29.3	23.4 ± 5.3

[a]Abbreviations: k_a, association rate; k_d, dissociation rate; K_D, dissociation constant

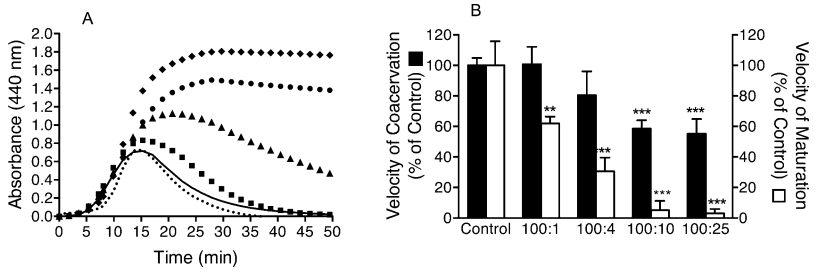

Figure 4. Effect of EBP on the self-assembly of tropoelastin. (A) EBP alters the self-assembly characteristics of recombinant human tropoelastin (hTE, 6.25 μM) in a concentration-dependent manner. hTE:EBP molar ratios of 100:0 (—), 100:1 (■), 100:4 (▲), 100:10 (●), and 100:25 (◆) were used, and a hTE:denatured EBP molar ratio of 100:4 (···) was used. EBP was denatured by being boiled in 0.1% SDS and 50 mM β-mercaptomethanol for 5 min. (B) Effect of EBP on the calculated velocities of coacervation (black bars) and maturation (white bars) of hTE (6.25 μM). Velocities of coacervation and maturation for hTE were normalized to values for a 100:0 hTE:EBP molar ratio. Mean ± SD (n = 3). **p < 0.01; ***p < 0.001.

Table 2. Effects of EBP and FKBP65 on the Coacervation Temperature of hTE [mean ± SD (n)]

	100:0	100:1	100:4	100:10	100:25
EBP	30.4 ± 0.8 (8)	31.8 ± 1.6 (3)	30.5 ± 1.6 (6)	31.3 ± 1.6 (6)	30.8 ± 1.8 (3)
FKBP65	31.3 ± 1.8 (5)	–	33.0 ± 0.4 (3)	33.2 ± 0.5 (5)[a]	33.1 ± 0.5 (5)[a]

[a]p < 0.05 vs control (100:0).

Molecular Interactions of Tropoelastin with EBP and FKBP65. Molecular interactions between tropoelastin and these two proteins were investigated by surface plasmon resonance analysis, with tropoelastin immobilized on the chip and exposed to different concentrations of EBP or FKBP65 in the flow-through solution. Both recombinant proteins showed strong, concentration-dependent interactions with tropoelastin (Figure 3). Analysis of the kinetics of these interactions (Table 1) indicated a dissociation constant (K_D) of 80.9 nM for the EBP−tropoelastin interaction and a weaker interaction between tropoelastin and FKBP65, with a K_D of 361.3 nM. In both cases, the association and dissociation curves were fit reasonably well by the Langmuir model, as indicated by the values for χ^2.

Effects of EBP and FKBP65 on the Self-Assembly of Tropoelastin. Self-assembly of tropoelastin and other elastin-like proteins is a two-stage process, the first of which, commonly termed coacervation, consists of the formation of small, protein-rich droplets resulting in a colloidal suspension whose turbidity can be monitored as the absorption at 440 nm.[17,53] Initiation of this phase transition takes place sharply, and the coacervation temperature is highly reproducible and dependent both on the concentration and on the sequence of the elastin-like protein, as well as on solution conditions, including the ionic strength.[2,18,19,54] Subsequently, if the colloidal suspension is held above the coacervation temperature, the suspension enters a second stage called maturation, during which turbidity decreases. We have shown that this decrease in turbidity corresponds either to growth in droplet size by coalescence or to clustering of the droplets.[17,53,55] Moreover, we have demonstrated that the presence of some matrix-associated proteins proposed to influence elastic fiber formation *in vivo* has a profound effect, particularly on this maturation stage of self-assembly.[17] Here, using methods described in detail previously,[17] we determined the effects of EBP and FKBP65 on the parameters of self-assembly of tropoelastin, including the coacervation temperature, and velocities of the coacervation and maturation stages.

EBP had a significant, concentration-dependent effect on the self-assembly of tropoelastin (Figure 4A). This effect could be seen at molar ratios as low as 100:1 (tropoelastin:EBP) and was particularly effective for decreasing the velocity of maturation of the coacervate (Figure 4B), although the velocity of coacervation was also affected at higher molar ratios. In contrast, the coacervation temperature of tropoelastin (approximately 31 °C under the conditions used) was unaffected at any of the molar ratios (Table 2). This effect of EBP was abolished after denaturation of the protein (Figure 4A). However, although lactose- and galactose-containing glycosaminoglycans have been reported to interfere with the binding of tropoelastin to EBP,[23,24] neither lactose (30 mM) nor chondroitin sulfate A (200 μg/mL) altered the effect of EBP on self-assembly of tropoelastin, as measured by these parameters (data not shown).

Recombinant FKBP65 had a similar, concentration-dependent effect on the self-assembly of hTE (Figure 5A), although again the coacervation temperature of hTE was either unaffected or affected only slightly at higher molar ratios of FKBP (Table 2). In the case of FKBP65, both the velocity of coacervation and the velocity of maturation were significantly affected, although this effect was only apparent at higher molar ratios compared to the effect of EBP. FKBP12, a FKBP family protein with a single FK506 binding domain,[52] at a molar ratio of 100:10 (hTE:FKBP12) had no effect on the coacervation of tropoelastin (Figure 5A). Neither FK506 nor rapamycin, each an inhibitor of the peptidyl-prolyl isomerase activity of

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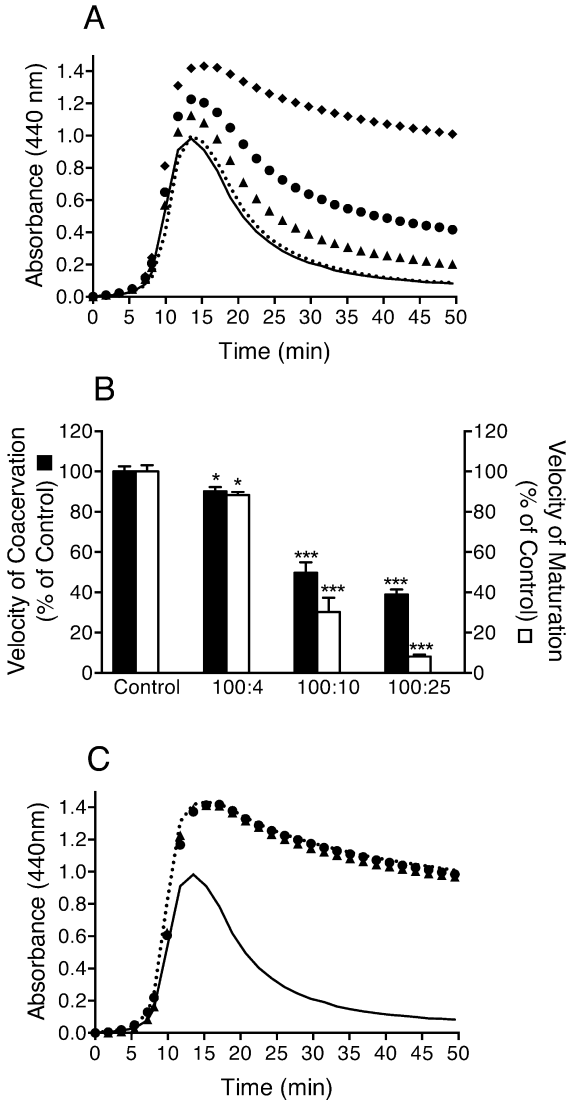

Figure 5. Effect of FKBP65 on the self-assembly of tropoelastin. (A) FKBP65 alters the self-assembly characteristics of recombinant human tropoelastin (hTE, 6.25 μM) in a concentration-dependent manner. hTE:FKBP65 molar ratios of 100:0 (—), 100:4 (▲), 100:10 (●), and 100:25 (◆) were used, and a hTE:FKBP12 molar ratio of 100:10 (···) was used. (B) Effect of FKBP65 on the calculated velocities of coacervation (black bars) and maturation (white bars) of hTE (6.25 μM). Velocities of coacervation and maturation were normalized to values for a hTE:FKBP65 molar ratio of 100:0. Mean ± SD (n = 3). *p < 0.05; ***p < 0.001. (C) Effects of FKBP65 on the coacervation of 6.25 μM hTE, at a hTE:FKBP65 molar ratio of 100:25 (···), are not inhibited by 10 μM FK506 (●) or 10 μM rapamycin (▲). The control is 6.25 μM hTE in the absence of FKBP65, at a hTE:FKBP65 molar ratio of 100:0 (—).

FKBP65, altered the effect of FKBP65 on the kinetics of hTE self-assembly (Figure 5C).

Peptidyl-prolyl Isomerase Activity of FKBP65. FKBP65 is a member of a family of isomerases that have the enzymatic activity to interconvert *cis* and *trans* configurations of proline residues in proteins.[37,38,40] We therefore investigated our recombinantly expressed FKBP65 for this activity and to determine whether such activity was correlated with effects on the self-assembly of hTE. The assay method for peptidyl-prolyl isomerase (PPI) activity is described in Experimental Procedures. FKBP65 exhibited significant, dose-dependent

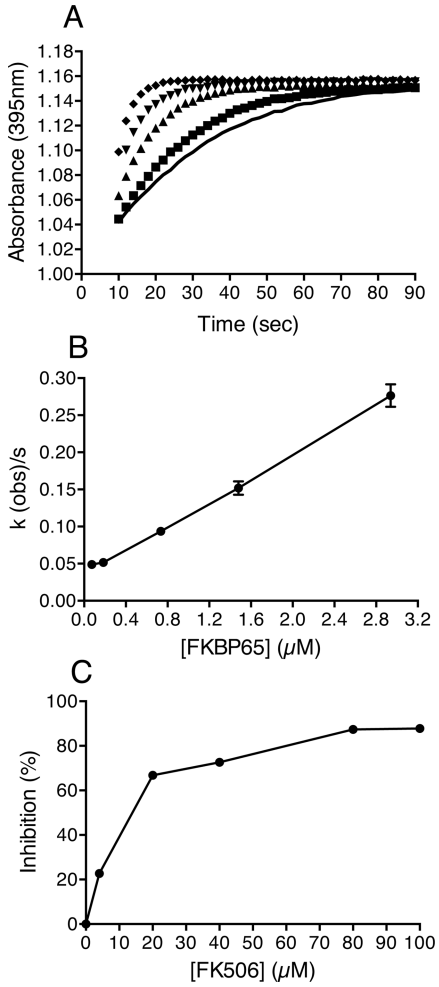

Figure 6. Peptidyl-prolyl isomerase (PPI) activity of FKBP65. (A) Recombinant human FKBP65 demonstrates PPI activity for substrate *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide in a concentration-dependent manner. FKBP65 concentrations of 0 (—), 0.18 (■), 0.74 (▲), 1.5 (▼), and 2.9 μM (◆). (B) Effect of FKBP65 concentration on the rate constant of peptidyl-prolyl isomerase activity [mean ± SD (n = 3)]. Calculated K_{cat}/K_m value of 82.5 mM^{-1} s^{-1}. (C) Concentration-dependent inhibition of the PPI activity of 0.74 μM FKBP65 by FK506 (representative curve from two experiments). Similar inhibition characteristics were seen for rapamycin.

PPI activity (Figure 6A). A plot of the observed rate constant (k_{obs}) versus FKBP65 concentration (Figure 6B) gave a K_{cat}/K_m ratio of 82.5 mM^{-1} s^{-1}. Under the same experimental conditions, the K_{cat}/K_m of FKBP12 was determined to be 170 mM^{-1} s^{-1} (data not shown). These values are comparable to catalytic activities previously reported for FKBP65 from chicken and mouse, and for human FKBP12.[37,40,51] FK506 was able to almost completely inhibit the isomerase activity of FKBP65 in a dose-dependent manner (Figure 6C). A similar inhibitory effect was seen for 20 μM rapamycin (data not shown).

Effect of FKBP65 Multimer Formation on the PPI Activity and Self-Assembly of Tropoelastin. As shown in Figure 1B, Western blotting of the purified recombinant FKBP65 revealed two minor bands at higher molecular masses in addition to the major band at the expected molecular mass. These higher-molecular mass bands were not present under reducing conditions, suggesting formation of disulfide-linked multimers. The population of these higher-molecular mass

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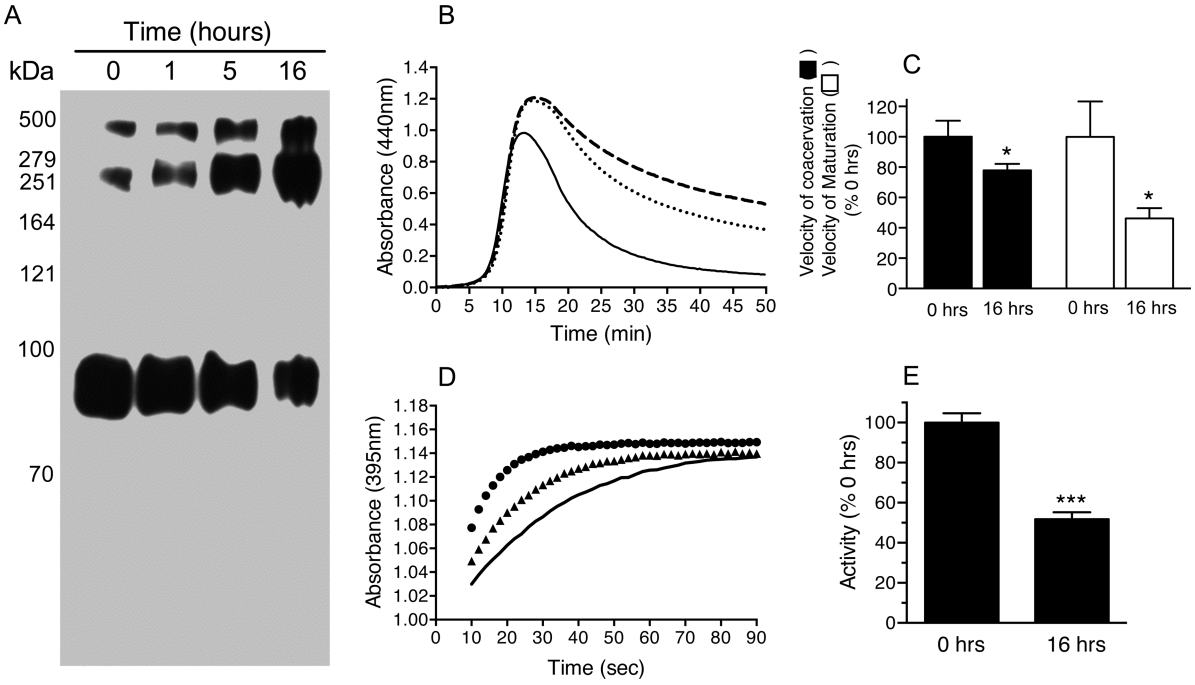

Figure 7. FKBP65 forms multimers with differential effects on the self-assembly of human tropoelastin (hTE) and peptidyl-prolyl isomerase (PPI) activity. (A) Time-dependent formation of FKBP65 multimers during incubation at 37 °C in 50 mM NaH_2PO_4 (pH 8.0) containing 300 mM NaCl. Bands represent various incubation times and are generated by Western blotting using a monoclonal anti-mouse FKBP65 antibody. (B) Effect of multimer formation on the modulation of self-assembly of hTE (6.25 μM). hTE:FKBP65 molar ratios of 100:0 (—), 100:10 with a 0 h incubation (···), and 100:10 with a 16 h incubation (- - -). (C) Effect of multimer formation (incubation time) on the velocities of coacervation and maturation of 6.25 μM hTE. Velocities of coacervation (black bars) and maturation (white bars) for hTE were normalized to values for a hTE:FKBP65 molar ratio of 100:10 with a 0 h incubation. Mean ± SD (n = 3). *p < 0.05. (D) Effect of multimer formation on PPI activity. Control [0 μM FKBP65 (—)], 0.74 μM FKBP65 with a 0 h incubation (●), and 0.74 μM FKBP65 with a 16 h incubation (▲). (E) Decreased PPI activity of 0.74 μM FKBP65 after incubation at 37 °C for 16 h, normalized to the activity at 0 h. Mean ± SD (n = 3). ***p < 0.001.

bands increased with incubation time at 37 °C in 50 mM NaH_2PO_4 (pH 8.0) containing 300 mM NaCl (Figure 7A), and FKBP65 incubated for 16 h at 37 °C demonstrated an enhanced effect on the coacervation of hTE (Figure 7B), decreasing both the velocity of coacervation and the velocity of maturation at molar ratios of 100:10, compared to unincubated FKBP65 (Figure 7C). In contrast, incubation of FKBP65 at 37 °C for 16 h significantly reduced its PPI activity relative to that of nonincubated FKBP65 (Figure 7C,D), indicating independent and differential effects of preincubation on these two activities.

Effect of EBP and FKBP on the Morphology of Tropoelastin Coacervate Droplets. Coacervation of recombinant human tropoelastin was visualized microscopically as the spontaneous formation of colloidal droplets when the solution temperature was held above the coacervation temperature. We have previously reported that such coacervate droplets normally grow by coalescence and are stabilized at a final diameter in suspension after approximately 60 min.[17,53] Under the conditions of this assay, coacervate droplets of human tropoelastin, upon contacting the bottom of the chamber, spread to coat the glass surface (Figure 8A), indicating substantial residual fluidity of the surface of the droplet. However, the addition of either FKBP65 or EBP to the coacervation solutions at a molar ratio of 10:1 (hTE:FKBP65 or hTE:EBP) resulted in a stabilization of the droplet surface and preservation of droplet morphology, even after contact with the bottom of the chamber (Figure 8B,C).

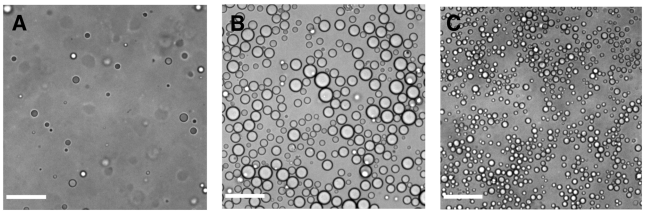

Figure 8. Effect of EBP and FKBP65 on the morphology of coacervation droplets of human tropoelastin (hTE). Microscopy details are given in Experimental Procedures. Images were taken 60 min postcoacervation and are focused on the bottom surface of the chamber: (A) hTE only (25 μM), (B) hTE:FKBP65 molar ratio of 25:2.5, and (C) hTE:EBP molar ratio of 25:2.5. The scale bar represents 31 μm.

■ DISCUSSION

The overwhelmingly nonpolar character of tropoelastin makes it susceptible to self-aggregation in polar environments. Indeed, such self-aggregation has been suggested to be an important early stage in the extracellular assembly of the elastic matrix.[3,11,20−22] Self-assembly of tropoelastin and elastin-like polypeptides involves a liquid−liquid phase separation called coacervation, consisting of the formation of protein-rich colloidal droplets. *In vitro*, these droplets initially grow rapidly by coalescence to approximately 1 μm in diameter and then continue to grow more slowly, usually to a limiting size, followed by clustering or semifusion of droplets into branched arrays or networks.[1,2,17,53] There is good evidence, at least *in vitro*, that other matrix-associated proteins such as fibrillins,

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fibulins, and MAGPs, all of which have been postulated to influence elastic matrix formation and architecture, modulate the growth and clustering of coacervate droplets, likely by affecting their surface properties.[17] While the most common method for initiating coacervation in solutions of tropoelastin is by increasing the solution temperature, in the *in vivo* situation this initiation event is likely triggered by elevated concentration.

The susceptibility of tropoelastin to self-aggregation is also an important consideration in the intracellular environment, as the newly synthesized protein moves through the endoplasmic reticulum and Golgi compartments before being secreted into the extracellular environment. This would especially be the case during stages of development when laying down of the extracellular elastic matrix is rapid, and local concentrations of the tropoelastin within intracellular compartments could increase to levels that initiate coacervation.

Two proteins, the so-called elastin binding protein (EBP) and FKBP65, a member of the immunophilin family of peptidyl-prolyl isomerases, have been previously suggested to function as intracellular chaperones for tropoelastin.[31−34] Because EBP is produced as a splice variant of β-galactosidase, it might be expected that knockout of the β-galactosidase gene would result in a phenotype affecting elastin-rich tissues such as large arteries and lung. However, such effects have not been reported, at least in a mouse model.[56] In contrast, both human Morquio B disease and infantile GM1 gangliosidosis have been reported to include impaired elastin assembly as a phenotypic consequence.[57] The predominant phenotype reported for mutations in the gene expressing FKBP65 is a severe form of osteogenesis imperfecta,[42−45] although more subtle effects on tropoelastin secretion and elastin assembly have not been ruled out. In all of these cases, other known chaperones such as BiP, which has also been identified in association with tropoelastin in the ER,[33] may at least partially compensate for deficiencies in EBP or FKBP65 with respect to intracellular trafficking of tropoelastin. While evidence had been published for FKBP65 modulating coacervation of tropoelastin,[36] the effects described depended on unusual coacervation conditions at very low concentrations of tropoelastin and the FKBP65 lacked post-translational modifications.

In the experiments reported here, both EBP and FKBP65 were expressed in a mammalian cell system, ensuring appropriate post-translational processing. Indeed, the FKBP65 expressed was demonstrated to be fully functional in terms of its peptidyl-prolyl isomerase activity. Circular dichroism data indicated that EBP was thermally stable, with structural melting taking place only at temperatures of >60 °C. In contrast, the structure of FKBP65 was thermally labile, with a melting temperature of approximately 45 °C. Interestingly, this is comparable to previous thermal melt data for FKBP65 in the absence of post-translational modifications[36] and is consistent with general suggestions for increased structural lability in chaperone proteins.[58] A practical consequence of the thermal lability of FKBP65 was that maximal temperatures in coacervation experiments were limited to 42 °C. Both EBP and FKBP65 showed significant binding to human tropoelastin, with dissociation constants in the nanomolar range, although the strength of binding of tropoelastin to EBP was >4-fold greater than to FKBP65.

We have previously established parameters for *in vitro* quantitation of effects of other proteins on the process of coacervation of recombinant elastin-like proteins and applied these to matrix-associated proteins thought to influence the extracellular assembly of the elastic matrix. These include the temperature at which the initial phase separation takes place (coacervation temperature), the rate of colloidal droplet formation (velocity of coacervation), and the rate of droplet growth or clustering (velocity of maturation).[17] Here these methods have been used to evaluate the influence of EBP and FKBP65 on the coacervation of human tropoelastin.

Consistent with previously reported results for extracellular matrix-associated proteins,[17] FKBP65 or EBP had little or no measurable effect on the coacervation temperature of tropoelastin, with the exception of FKPB65 at higher molar ratios where the coacervation temperature was slightly elevated. However, EBP and FKBP65 had significant, concentration-related effects on both the velocity of coacervation and the velocity of maturation of tropoelastin. For both proteins, this effect was more pronounced on the velocity of maturation, and significant effects could be detected at molar ratios of ≤100:4. Furthermore, EBP appeared to be more potent than FKBP65 in affecting these parameters. In the case of EBP, denaturation by boiling in 0.1 M SDS and 50 mM β-mercaptoethanol completely inhibited any effect on coacervation. In the case of FKBP65, FKBP12, at comparable molar ratios, had essentially no effect on coacervation. Neither rapamycin nor FK506, each an established inhibitor of PPI activity of FKBP65, had any ability to inhibit the effect of FKBP65 on the coacervation of tropoelastin.

Because of the abundance of proline residues (approximately 12%) in human tropoelastin, and the potential impact of *cis*−*trans* isomerization of proline residues on the structure of tropoelastin, it was suggested that the role of FKBP65 as a chaperone might be related to the PPI activity of this protein. Our results clearly indicate that this is not the case for several reasons. In the first place, FKBP12, which also possesses PPI activity, had no effect on the coacervation of tropoelastin. Second, rapamycin and FK506, inhibitors of PPI activity, had no effect on the ability of FKBP65 to modulate coacervation. Third, oligomerization of FKBP65, which takes place upon incubation at 37 °C, enhanced the effect of FKBP65 on coacervation but reduced PPI activity. Together, these results suggest that the ability of FKBP65 to slow formation and maturation of coacervate droplets of tropoelastin is not related to its PPI activity.

The morphology of the coacervate droplets formed in the presence and absence of EBP or FKBP65 was also followed microscopically. The velocity of maturation of coacervated tropoelastin has been suggested to be related to the rate of growth of colloidal droplets by coalescence, increasing the size of these droplets and decreasing light scattering as detected by turbidity.[1,2,17,53] While decreased turbidity can also be the result of clustering of droplets,[17] in this case no clustering was seen. Coalescence of smaller droplets to form a single larger droplet depends on their surface fluidity, with increased surface stability resulting in inhibition of coalescence and smaller droplet size. Similar to the results previously described for the effects of extracellular matrix-associated proteins on the coacervation of tropoelastin,[17] the low molar ratios of EBP and FKBP65 sufficient to produce these effects are suggestive of a role of these proteins in stabilizing the surface of the coacervate droplet and slow growth. This is consistent with our observations that, in the absence of FKBP65 or EBP, the stability of droplets of tropoelastin was insufficient to prevent spreading when they fell to the bottom of the microscope chamber. In contrast, in the presence of EBP, coacervate

droplets retained their shape, even after touching the bottom surface of the chamber. It also should be noted that the smaller size of the droplets formed in the presence of EBP as compared to FKBP65 implies a greater degree of surface stabilization, consistent with the more potent effects of EBP on the velocity of maturation, as well as the increased strength of binding of EBP to tropoelastin in binding studies.

Interactions with a series of "companion" proteins appear to be important throughout the passage of tropoelastin from its initial secretion into the endoplasmic reticulum to its final assembly in the elastic fibres of the extracellular matrix. Unlike most other proteins, tropoelastin does not fold into a stable, fixed conformation but rather must retain a conformationally flexible and intrinsically disordered state.[12−14] Thus, the classical role of an intracellular chaperone to assist in proper protein folding would not seem to be required. However, because of its unusual nonpolar character, tropoelastin is highly susceptible to self-aggregation in its native state.

The data presented here provide clear evidence that both FKBP65, confined to the endoplasmic reticulum,[34] and EBP, in Golgi and post-Golgi compartments,[31,32] could play a role in attenuating premature intracellular self-aggregation of tropoelastin. The stoichiometry of their effects suggests that, rather than absolutely preventing intracellular coacervation of tropoelastin, these proteins may function by stabilizing the surface of coacervate droplets and inhibiting their growth, somewhat analogous to antifreeze proteins that act not to prevent the formation but rather to limit the growth of ice crystals.[59−61] The process of transfer of such packets of tropoelastin from FKBP65 in the endoplasmic reticulum to EBP at the ER−Golgi interface is unknown, although the greater affinity of tropoelastin for EBP compared to its affinity for FKBP65 observed here may be one factor facilitating this transfer. EBP has been shown to accompany tropoelastin as it is secreted into the extracellular compartment, at which point tropoelastin forms associations with other matrix proteins, including fibrillins, fibulins, and MAGPs, guiding its orderly transfer and cross-linking to the growing elastic fiber and regulating the final architecture of the extracellular elastic matrix.[3,5,6,9−11] While many details of the production, intracellular trafficking, secretion, and final assembly of this unusual protein remain to be determined, the data presented here contribute to the growing body of evidence of the importance of both intracellular and extracellular associations in the assembly of tropoelastin into the extracellular elastic matrix.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS

ELP, elastin-like polypeptide; EBP, elastin binding protein; ER, endoplasmic reticulum; hTE, human tropoelastin; IPTG, isopropyl β-D-1-thiogalactopyranoside; CD, circular dichroism; SPR, surface plasmon resonance; SDS, sodium dodecyl sulfate; PPI, peptidyl-prolyl isomerase; SD, standard deviation.

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