Domain 26 of Tropoelastin Plays a Dominant Role in Association by Coacervation*

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The temperature-dependent association of tropoelastin molecules through coacervation is an essential step in their assembly leading to elastogenesis. The relative contributions of C-terminal hydrophobic domains in coacervation were assessed. Truncated tropoelastins were constructed with N termini positioned variably downstream of domain 25. The purified proteins were assessed for their ability to coacervate. Disruption to domain 26 had a substantial effect and abolished coacervation. Circular dichroism spectroscopy of an isolated peptide comprising domain 26 showed that it undergoes a structural transition to a state of increased order with increasing temperature. Protease mapping demonstrated that domain 26 is flanked by surface sites and is likely to be in an exposed position on the surface of the tropoelastin molecule. These results suggest that the hydrophobic domain 26 is positioned to play a dominant role in the intermolecular interactions that occur during coacervation.

Coacervation of tropoelastin molecules is a crucial step in the formation of the elastic fiber. It is an entropically driven, inverse temperature transition caused by the interaction of the hydrophobic domains in the molecule. The coacervate is the favored form of tropoelastin under conditions found in the extracellular matrix (1), and its water content of 60% is similar to that found in mature elastin (2). When viewed by electron microscopy, coacervates of α-elastin, tropoelastin, and polymeric models of the hydrophobic repeats appear as 5-nm wide, filamentous structures (3–5). Filaments found in the coacervates of tropoelastin have been shown to associate laterally to form large aggregates with the amorphous appearance typical of tissue elastin (6). During elastogenesis, coacervation concentrates and aligns tropoelastin molecules prior to cross-linking by the enzyme lysyl oxidase (7). Coacervation results in an increase in the amount of α-helical structure, and it has been suggested that this can be attributed to the alanine-rich cross-linking domains (8, 9).

Coacervation is finely tuned to the conditions of the extracellular matrix, with optimal coacervation occurring at 150 mM NaCl, 37 °C, and pH 7–8 (1). The coacervation characteristics of polymeric models of the repeat peptides VPGG, VPGVG, and APGVGV have been extensively studied (10). Amino acid sequence is an important factor: poly(VPGG) and poly(VPGVG) coacervate reversibly, whereas poly(APGVGV) precipitates irreversibly (11). Polymeric models of elastin-like peptides, which lack the PG dipeptide, such as poly(VGGLG), are unable to coacervate (12). The formation of β-turns is critical in the aggregation of these polymers (10, 12, 13). In the PG-containing sequences, type II β-turns are formed with PG that are the corners of the bend. Proline stabilizes the β-turns of these domains because its φ angle is fixed at −60°, which is typical for an ideal β-turn (14). Replacing proline with glycine would increase the flexibility of the molecule and reduce the stability of the β-turn. In tropoelastin, PG-containing hydrophobic repeats are found in the larger hydrophobic domains (up to 55 residues in length) near the center of the molecule, such as those encoded by exons 18, 20, 24, and 26. Proline-depleted hydrophobic domains, such as those encoded by exons 28, 30, and 32, are smaller (20–25 residues).

Despite the extensive work done on polymeric models of the hydrophobic repeats, the roles of the individual hydrophobic domains in the native tropoelastin molecule remain unclear. Protease cleavage provides a direct probe of protein conformation, because it relies on direct interactions between the enzyme and individual peptide bonds in its target protein (15, 16). We describe here kallikrein protease mapping of human tropoelastin that explores the surface composition of folded tropoelastin. Recognition of exposed residues is limited to two major sites that flank a 41-amino acid domain encoded by exon 26. We have previously shown that C-terminally truncated forms of tropoelastin, which contained as much as the first 25 domains from the N terminus, displayed deficient coacervation under physiological conditions of salt concentration and temperature (17). Domain 26 is essential for the association of truncated tropoelastin molecules at 37 °C with a concomitant increase in β-structure. We propose that domain 26 is positioned to play a dominant role in tropoelastin assembly for elastogenesis.

EXPERIMENTAL PROCEDURES

Protein Preparation—SHELΔ26A, a human tropoelastin isoform lacking the 26A domain, was obtained by overexpression from the plasmid pSHELFΔ26A in Escherichia coli BL21(DE3). pSHELFΔ26A was derived from the plasmid pSHELF (18) by deletion of the sequence encoding the 26A domain (19). The purification method for SHELΔ26A was essentially the same as that described previously for SHEL (1), with minor changes to the reverse phase HPLC step. After lyophilizing from 50 mM ammonium acetate, pH 5.0, the protein was loaded onto a reverse phase column (Delta-Pak C-18, 25 mm × 100 mm, Waters) and eluted along a linear gradient of 30–80% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid over 60 min.

SHELΔ26C and SHELΔ26A:C (Fig. 1a) consist of domains 26–36 of human tropoelastin. SHELΔ26A:C is identical to SHELΔ26C except that it lacks the 26A domain. SHELΔ26C and SHELΔ26A:C were constructed with N termini positioned variably near the center of the molecule, such as those encoded by exons 28, 30, and 32, are smaller (20–25 residues).

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1 The abbreviations used are: SHEL and SHELΔ26A, recombinant human tropoelastin isoforms; HPLC, high pressure liquid chromatography; TFE, trifluoroethanol.
obtained by respective digestion of SHEL and SHEL26A with porcine pancreatic kallikrein (EC 3.4.21.3; Sigma). Digestion cleaves human tropoelastin on the C-terminal side of the final arginine residue of domain 26 (Table I). 10 mg/ml solutions of tropoelastin were prepared in 50 mM sodium phosphate, pH 7.8, and porcine pancreatic kallikrein was added to a concentration of 0.1 unit/ml. Digestion was at 37 °C for 2 h. The digests were then loaded onto a reverse phase column (Delta-Pak C-18, 25 mm x 100 mm, Waters) and eluted along a linear gradient of 30–80% (v/v) acetonitrile. SHEL26A/C eluted at 46% acetonitrile and SHEL26C eluted at 44% acetonitrile.

SHEL26.5C was prepared by annealing the oligonucleotides 5'-GGATCTGAGGCGGTCTCCGACCATGGAGCTCGTCGGCCTAATC-3' and 5'-GCCGAGGCCCAGGATCTTTGATGTTCCCTACTAC-3' and ligating them into the KpnI site of the plasmid pSHELF (18). The 604-base pair Ncol-BamHI fragment of the resulting construct was excised and inserted into the Ncol-BamHI-digested expression vector pET-3d (20). The resulting plasmid, pSHELK, was transformed into E. coli BL21(DE3) cells for expression of SHEL26.5C. Expression and purification conditions for SHEL26.5C were essentially the same as those described above for SHEL26A, except that expression was induced by the addition of isopropyl-1-thio-β-D-galactopyranoside to 1 mM instead of 0.04 mM. The resulting protein has an N terminus corresponding to the central region of domain 26 (Fig. 1b).

The isolated domain 26 peptide was prepared by amplifying the sequence corresponding to human exon 26 from the plasmid pSHELF26A using the primers 5'-GGATCTGAGGCGGTCTCCGACCATGGAGCTCGTCGGCCTAATC-3' and 5'-GGATCTTGATGTTCCCTACTAC-3'. The resulting 138-base pair polymerase chain reaction product was cloned into the vector pGEM-T Easy (Promega) and sequenced. The 132-base sequence corresponding to human exon 26 from the plasmid pSHELF was inserted into the central region of domain 26 (Fig. 1b). The isolated domain 26 peptide was prepared by amplifying the sequence corresponding to human exon 26 from the plasmid pSHELF26A using the primers 5'-GGATCTGAGGCGGTCTCCGACCATGGAGCTCGTCGGCCTAATC-3' and 5'-GGATCTTGATGTTCCCTACTAC-3'. The resulting 138-base pair polymerase chain reaction product was cloned into the vector pGEM-T Easy (Promega) and sequenced. The 132-base pair BamHI fragment containing the exon 26 sequence was subsequently excised from this clone and inserted into the BamHI site of the expression vector pGEX-2T (21). The resulting plasmid, pSHELEX26, was transformed into E. coli DH5α cells as a glutathione transferase (E. coli DH5α) using a Neslab RTE-111 waterbath to control the temperature of the cell. Scans of the peptide in water were obtained between 184 and 260 nm.

RESULTS

Protease Mapping of Tropoelastin—Increasing amounts of human plasma kallikrein added to SHEL resulted in the production of three major fragments around 45, 22, and 18 kDa as estimated by SDS-polyacrylamide gel electrophoresis (Fig. 2A). The major bands at 45 and 18 kDa were resistant to further degradation, whereas the 22-kDa fragment eventually disappeared. In the case of SHEL26A, the pattern of degradation was somewhat different with the 22- and 18-kDa fragments being replaced by a 15-kDa fragment (Fig. 2B). N-terminal sequencing of the fragments (Table I) showed that two sites within SHEL were susceptible to cleavage by human plasma kallikrein: the first occurring after Arg515 at the end of domain 25 and the second after Arg564 within domain 26A. The region of tropoelastin between these cleavage sites contains the entire domain 26 sequence (Fig. 2C).

Coacervation—Coacervation was measured by monitoring light scattering at 300 nm using a Cary 3 spectrophotometer and software as described previously (1). Protein samples were prepared in phosphate-buffered saline (10 mM sodium phosphate, pH 7.4, 150 mM NaCl). To determine the maximal rates of coacervation, first derivatives of the time courses of coacervation were obtained. The maximal rate of coacervation was defined as being the maximum slope of the coacervation curve. Circular Dichroism (CD) Spectroscopy—CD spectra of 100 μM solutions of the domain 26 peptide, in a 0.5-mm cell, were recorded on a Jasco J720 CD spectropolarimeter (Japan Spectroscopic Co. Ltd., Jap-
A comparison of the maximal rates for SHEL26(Δ26A)C and SHEL26A revealed that the coacervation of SHEL26(Δ26A)C was less favorable kinetically (Fig. 5). The maximal rate for SHEL26A was found to increase rapidly over a small temperature interval. SHEL26(Δ26A)C, however, showed less temperature sensitivity, with the maximal rates increasing more gradually with increasing temperature. By 60 °C, the rate for SHEL26(Δ26A)C was only 34% the rate of SHEL26A. This effect correlates with a greater level of cooperativity in the coacervation of the full-length molecule because of the presence of additional interacting hydrophobic domains.

**Effect of Domain 26A on Coacervation**—Domains 26 and 26A are encoded by consecutive exons. For intact tropoelastin isoforms, the temperatures of coacervation are slightly lower for SHEL26A than for SHEL (19), suggesting that domain 26A has an inhibitory effect. The effect of domain 26A was further examined by comparing SHEL26C, which contains domain 26A, with SHEL26(Δ26A)C, which lacks 26A (Fig. 4). SHEL26(Δ26A)C coacervated at 35 °C, but this did not proceed to completion until the temperature was ~60 °C. Coacervation of SHEL26C did not occur at temperatures below ~45 °C. Temperatures for SHEL26C were at least 10 °C higher than those required to reach the same extent using SHEL26(Δ26A)C, even though the concentration of SHEL26C used in the assay was higher than SHEL26(Δ26A)C. Domain 26A is a hydrophilic domain unique to human tropoelastin and is largely unstructured except for a single β-turn formed around the sequence REGD (22). Its effect on coacervation would be more obvious in the shorter fragments of tropoelastin studied here than in the full-length isoforms because it constitutes a larger proportion of the smaller molecule.

**Role of Domain 26**—Domain 26 is the largest hydrophobic domain in SHEL26C and SHEL26(Δ26A)C. SHEL26.5C is identical to SHEL26C, except that it lacks the N-terminal half of domain 26 (Fig. 1b). A comparison of the profiles of SHEL26.5C with those of SHEL26C and SHEL26(Δ26A)C (Fig. 4) showed that disruption of domain 26 disabled coacervation. This indicated a central role for domain 26 in the coacervation of SHEL26C and SHEL26(Δ26A)C and that the hydrophobic domains encoded by exons 28, 30, and 32 did not significantly contribute to the process. Isolated domain 26 peptide did not coacervate, revealing a contextual requirement. Solutions of the peptide, at concentrations up to 4 mg/ml, were examined at temperatures between 30 and 90 °C.

**Effect of Concentration on Coacervation of SHEL26(Δ26A)C**—As in the cases of full-length tropoelastin (1, 19), coacervation of SHEL26(Δ26A)C was found to be concentration-dependent (Fig. 6). At 2.0 mg/ml, no effect was observed at temperatures below 40 °C, and the extent of coacervation correlated with temperature. At 9.5 mg/ml, coacervation occurred readily between 30 and 35 °C. At this concentration, further increases in temperature caused sharper rises compared with the 2.0 mg/ml sample. Between 46 and 50 °C, coacervation of the 2.0 mg/ml sample rose by 0.5 absorbance units, whereas between 35 and 37 °C, the extent of coacervation of the 9.5 mg/ml sample increased by 0.82 absorbance units. This sharpening of the curves suggested that this was a cooperative process for SHEL26(Δ26A)C.

**Effect of Temperature on Domain 26**—CD spectra of the peptide corresponding to domain 26 (Fig. 7a) revealed that it became more structured with increasing temperature. At 20 °C, the spectrum was characterized by an intense band of ~8220 deg cm² dmol⁻¹ at 195 nm, and a less intense band of ~620 deg cm² dmol⁻¹ centered at 227 nm. With increasing temperature, the band at 195 nm changed to ~6350 deg cm² dmol⁻¹ at 50 °C, indicating a transition to a more ordered state. The band at 227 nm also changed to ~1200 deg cm² dmol⁻¹ at 80 °C.
using diverse synthetic models (10, 11, 13). Polymeric models consisting of the VPGVG and VPGG repeats coacervate, whereas those consisting of APGVGV precipitate irreversibly (10). Proline-depleted sequences lacking the PG dipeptide, such as VGGVG, are unable to coacervate (12, 25) but play an important role in the elasticity of the elastic fiber (14). Polymers of the human W4 sequence, which corresponds to the domain encoded by exon 18, are elastomeric and are able to coacervate at physiological temperature (26). The contextual roles of individual hydrophobic domains in the intact tropoelastin molecule and their associations with other hydrophobic domains are unclear.

Protease mapping was used to examine if a specific hydrophobic region is located on the surface of tropoelastin. A preferred candidate for domain mapping in tropoelastin is plasma kallikrein, which is the major serum protease capable of degrading unprotected tropoelastin (27). Human tropoelastin was degraded by human plasma kallikrein into discrete bands of 45, 42, and 18 kDa. To map sites precisely, fragments were N-terminally sequenced and assigned to regions of SHEL. One site immediately C-terminal to Arg515 was common to digestion by human serum, human plasma kallikrein, porcine pancreatic kallikrein, and thrombin (data not shown). The second major location cleaved by plasma kallikrein was in 26A as identified by sequencing. Remarkably, these exposed regions in SHEL flank a hydrophobic domain corresponding to that encoded by exon 26. A straightforward interpretation of the data is that the bracketed 41-amino acid domain 26 is exposed on the surface of tropoelastin. Its hydrophobic content precludes cleavage by kallikrein. Domain 26, AAAGLGAGI, is marked by four nonapeptide repeats punctuated by Pro residues and describes the longest unit repeat in this molecule (18). Polymers of shorter similar structures are capable of association by coacervation because of strong interactions with bulk water (10, 11). Coacervation is an important step in the alignment and incorporation of tropoelastin molecules into the growing elastic fiber where it constitutes the major stage of elastin biosynthesis. We wanted to know if domain 26 plays a dominant role in the coacervation of tropoelastin at 37 °C. As this region is in the C-terminal quarter of the molecule, we examined the coacervation of derivatives of tropoelastin with variable N termini and a shared C terminus.
SHEL26C and SHEL26(Δ26A)C consist of domains 26–36
with or without domain 26A. Both forms were found to coacervate. Coacervation of SHEL26(Δ26A)C was less efficient than the coacervation of the full-length tropoelastin isoform SHEL26A. Measurements of SHEL26(Δ26A)C coacervation showed that this molecule was less temperature-sensitive than SHEL26A, which showed a sharpening of its profile near the transition point. This effect is attributed to a cooperative process during coacervation with the induction of conformational changes. Similar observations were made in studies of α-elastin, poly(VPGG), and poly(APGVG) (11, 13, 28). In contrast, temperature profiles for the polyhexapeptide poly(APGVGV), which does not coacervate, are characterized by a decrease in transition temperature at increasing concentrations with little or no increase in the steepness of the transition (11). The concentration dependence seen in the steepness of the coacervation curves of SHEL26(Δ26A)C confirmed the cooperative nature of the interaction.

Domain 26A is adjacent to domain 26 but is often spliced out in tropoelastin. SHEL26C and SHEL26(Δ26A)C are identical except for the presence of domain 26A (Fig. 1a). Comparison of the behavior of SHEL and SHEL26A (11, 19) indicated that the presence of 26A in intact tropoelastin had little effect. Domain 26A is unique to human tropoelastin (29) and is unusual in that it contains a large proportion of charged and polar residues compared with other tropoelastin domains. Of the 14 Ser residues in the tropoelastin amino acid sequence, 8 are found within this region (18). The unstructured, hydrophilic nature of this domain would be expected to increase the temperature at which coacervation occurs by increasing the hydration of the polypeptide (30). The observed difference in the performance of SHEL26C and SHEL26(Δ26A)C would have been more obvious than between SHEL and SHEL26A because of the larger relative change in size of the smaller molecules.

SHEL26C and SHEL26.5C differ by deletion of the first 19 amino acids of domain 26. This was sufficient to abolish coacervation. Preliminary experiments reveal no coacervation for SHEL27C, which consists of domains 27–36 (data not shown). Domain 26 has the longest repeat unit size of any of the repetitive hydrophobic domains of tropoelastin and consists of 4 repeats of the sequence XGXXGXXGVP, where X is either A, L, V, or I. The formation of type II β-turns at the PG segments would be expected on the basis of structural studies of poly(VPGG), poly(VPGVG), and poly(APGVG) (11). Domain 26 may form about 1.5 turns of a loose β-spiral of the type displayed by the polynonapeptide poly(VPGG) (25). Among mammalian tropoelastins, domain 26 is relatively well conserved (31), suggesting that it may have an important role. It is adjacent to cross-linking domain 25, which forms a desmosine cross-link with the lysines of domain 19 (32), in a potential role aligning tropoelastin molecules prior to cross-linking.

The lack of coacervation displayed by SHEL26.5C suggests that domain 26 is required for the coacervation of SHEL26C and that the hydrophobic domains encoded by exons 28, 30, and 32 are unlikely to initiate coacervation. These domains are smaller than the central hydrophobic domains, such as those encoded by exons 18, 20, and 24, and lack the regularly repeating proline residues required for the formation of a β-spiral (14, 33). It was not clear whether domains 28, 30, and 32 are involved in intermolecular interactions after a cooperative process after the coacervation of SHEL26C and SHEL26(Δ26A)C is initiated by domain 26.

CD spectroscopy of domain 26 revealed a disordered molecule with an increase in structure at elevated temperatures. Similar conformational changes were observed for peptides based on the sequence VPGVG (34). CD spectra of β-sheets often display a negative band in the 210–220 nm region and a positive band in the 190–200 nm region of the spectrum (23, 24). The temperature-induced transition seen for domain 26 probably represented an increase in β-structure. Measurements of the ellipticity at 200 nm between 10 and 45 °C indicated that the transition reached a plateau at about 37 °C, which supports a model of the importance of domain 26 in coacervation at physiological temperature. Truncated forms of tropoelastin comprising the N-terminal 25 domains of the protein display coacervation only above 37 °C (19), suggesting that one or more of the C-terminal domains are involved in coacervation. In the work presented here, SHEL26(Δ26A)C coacervated readily at 37 °C. In combination with the CD data demonstrating a conformational change in domain 26 by about 37 °C, these results support a model where domain 26 serves an important function in determining the behavior of intact tropoelastin at physiological temperature.

Domain 26 should be considered functionally as part of the larger molecules. Isolated domain 26 is unlikely to self-associate as there was no evidence of a concentration-dependent conformational change in our CD data. This lack of self-association was confirmed by sedimentation equilibrium analytical ultracentrifugation studies on the domain 26 peptide. TFE was used to determine how the secondary structure of domain 26 may be affected by the hydrophobic environment of the coacervate. TFE induces a conformational change in α-elastin similar to that seen during coacervation (35). We similarly found that TFE promotes a conformational change to a higher proportion of β-structure. We propose that domain 26 undergoes a temperature-induced conformational change that is enhanced by the hydrophobic environment of the coacervate. During coacervation of SHEL26C and SHEL26(Δ26A)C, interactions between domain 26 and domains 28, 30, and/or 32 may also occur. Domains 19 and 25 have been shown to be involved in the formation of a desmosine cross-link and that the polypeptide chains are aligned in an antiparallel arrangement (32). Thus, domain 26 is ideally positioned to allow it to interact hydrophobically with domain 18 in the intact tropoelastin molecule.

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