Hydrophobic Domains of Human Tropoelastin Interact in a Context-dependent Manner*

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Tropoelastin is the soluble precursor of elastin, the major component of the extracellular elastic fiber. Tropoelastin undergoes self-association via an inverse temperature transition termed coacervation, which is a crucial step in elastogenesis. Coacervation of tropoelastin takes place through multiple intermolecular interactions of its hydrophobic domains. Previous work has implicated those hydrophobic domains located near the center of the polypeptide as playing a dominant role in coacervation. Short constructs of domains 18, 20, 24, and a mutated form of domain 26 were largely disordered at 20 °C but displayed increased order on heating that was consistent with the formation of β-structures. However, their conformational transitions were not sensitive to physiological temperature in contrast to the observed behavior of the native domain 26. A polypeptide consisting of domains 17–27 of tropoelastin coacervated at temperatures above 60 °C, whereas individually expressed hydrophobic regions were not capable of coacervation.

We conclude that coacervation depends on the hydrophobicity of the molecule and, by inference, the number of hydrophobic domains. Tropoelastin mutants were constructed to contain a Pro → Ala mutation in domain 26, separate deletions of domains 18 and 26, and a displacement of domain 26. These constructs displayed unequal capacities for coacervation, even when they contained the same number of hydrophobic regions and comparable levels of secondary structure. Thus, the capability for coacervation is determined by contributions from individual hydrophobic domains for which function should be considered in the context of their positions in the intact tropoelastin molecule.

Elastin is a protein polymer that forms an integral part of the elastic fibers in the extracellular matrix. Following secretion to the cell surface, the soluble elastin precursor tropoelastin self-associates in a process called coacervation, which is an inverse temperature transition arising from multiple hydrophobic interactions (1). Studies using panels of synthetic elastin peptides indicate that the temperature at which the inverse temperature transition occurs is determined primarily by the hydrophobicity of the molecule (2–5), whereas for full-length tropoelastin, optimal coacervation is obtained under conditions mimicking those found in the extracellular matrix. Coacervation has been proposed to concentrate and align tropoelastin molecules for cross-linking, as a key step in elastogenesis (6).

During coacervation, tropoelastin undergoes a conformational transition from being predominantly unstructured to a more ordered state at both intramolecular and intermolecular levels (7–10). This is mainly because of the formation of β-structures, as the recurring PG sequences in hydrophobic domains form a series of type II β-turns that subsequently fold into β-spirals and twisted filaments (11). Ultrastructurally, tropoelastin coacervates are seen as parallel arrays of 5-nm wide filaments that are similar to the fibrous structure of mature elastin (12–14).

Domain 26, one of the large hydrophobic domains found in the central region of tropoelastin, plays an important role in coacervation. Domain 26 alone does not coacervate, but its presence is necessary for coacervation of truncated forms of tropoelastin that have N termini placed variably downstream of domain 25 (15). Other hydrophobic domains have also been implicated in coacervation. Identification of a putative cross-linking motif containing the hydrophilic domains 10, 19, and 25 has led to a model in which domains 19 and 25 are linked by a desmosine cross-link in an antiparallel fashion (16). This model may require direct interactions between domains 18 and 26 and between 20 and 24, which are the four largest PG-containing hydrophobic domains located near the center of the molecule (15). Together, these findings suggest a role for these hydrophobic regions in coacervation. The uniquely defined position of each region would enable specific pairing of hydrophobic domains during coacervation, and, by inference, the alignment of cross-linking sites.

The work presented here explores some of the properties of the central hydrophobic domains of tropoelastin and their contribution to coacervation in the context of position in the full-length molecule. Various tropoelastin constructs, including a naturally occurring isoform of tropoelastin (SHELJ26A), its mutant forms, and short forms, were studied with respect to their secondary structures and coacervation abilities (Fig. 1). One mutant contained a Pro → Ala mutation that was chosen because of the importance of Pro in the structures of elastin peptides (5, 17). Two other mutants were made to lack domains 18 and 26, and the last mutant contained a displaced domain 26. All constructs displayed similar CD spectra and contained approximately the same amounts of secondary structures. A greater capacity for intermolecular interactions correlated with more hydrophobic domains. Yet tropoelastin mutants with comparable hydrophobicity to the native form displayed decreased ability to coacervate. These results indicate that coacervation is not merely a result of random interactions of hydrophobic domains but that it is caused by multiple and specific interactions of individual hydrophobic domains of tropoelastin.
and SHELex26(P534A) (◦circles ◦), SHELex24 (◦triangles ◦), SHELex18 (◦squares ◦), SHEL17–27 corresponds to domains poelastin lacking domain 26A (18). SHEL18–(18,26A), and SHEL26A(P534A), SHEL(18,26A) is a 56.6-kDa mutant form of human tropoelastin lacking domains 26 and 26A but with domain 26 transferred to the C terminus of the molecule (Fig. 1). The deletion of a 129-bp sequence corresponding to exon 26 from human tropoelastin lacking domains 26 and 26A (Fig. 1), was performed by site-directed mutagenesis using the plasmid pSHELF26A as a template and the oligonucleotides 5′-CGG CCA AAG CAG GCA GCT GCC GGG CGC GCT GGC TGC TGC GAA-3′ and 5′-CCC TGC ACC TAC ACC CAG GCC TGC AAC ACC AAC-3′ corresponding to three stop codons at the 3′ end of the sequence, thus allowing for the data obtained from SHELex18, SHELex20, and SHELex24. QuikChange site-directed mutagenesis kit (Stratagene) using the plasmid pSHELF26A as a template and the oligonucleotides 5′-CGG CCA AAG CAG GCA GCT GCC GGG CGC GCT GGC TGC TGC GAA-3′ and 5′-CCG CTA AAT AGG TTT CAG TTA GGC TGC TGG TTG TGG TGT TGC AGG-3′. SHEL(26,26A) is a 56.1-kDa mutant form of human tropoelastin lacking domains 18 and 26A (Fig. 1). Deletion of a 150-bp sequence corresponding to exon 18 was performed by site-directed mutagenesis using the plasmid pSHELF26A as a template and the oligonucleotides 5′-CGG CTA AAT AGG TTT CAG TTA GGC TGC TGG TTG TGG TGT TGC AGG-3′ and 5′-GCC TCT TGG GGG GAT ACA AGC TAT TTA GCT GCC TCT GCC GCA GCT-3′. SHELex(26,26A) is a 56.6-kDa mutant form of human tropoelastin lacking domains 26 and 26A (Fig. 1). The deletion of a 129-bp sequence corresponding to exon 26 was performed by site-directed mutagenesis using the plasmid pSHELF26A as a template and the oligonucleotides 5′-TAT CCC CGG AAG CGG C-3′ and 5′-GCC TCT TGG GGG GAT ACA AGC TAT TTA GCT GCC TCT GCC GCA GCT-3′. SHELex(26,26A)+26 is a 60.0-kDa mutant form of human tropoelastin lacking domains 26 and 26A but with domain 26 transferred to the C terminus of the molecule (Fig. 1). The SHEL(26,26A)+26 coding sequence was produced by a two-step DNA manipulation. First, site-directed mutagenesis was performed to replace a 9-bp sequence corresponding to three stop codons at the 3′-end of the SHEL(26,26A) sequence with a 12-bp sequence corresponding to BamHI and EcoRI cloning sites using the oligonucleotides 5′-CGG GCC GCC GGG ACC GTGA AAT AAT TGC CTT TGG GCT CCT ACC AAG AAG CCA CCA CCA GCT GGG TTG TGC TGG TTG TGT TGC AGG-3′ and 5′-GCT TCT CTT CAG CCA CCA GAG GCC GCC ACC GCC GCT GCC ACC GCA GAG GAC-3′ and 5′-CCG CTG CTT CAG CCA GGC GCC GCC GCC GCT GCC TGG TGC ACC ACC AGC CGC GCT-3′.

SHEL26A(P534A) was achieved with the

1 The abbreviation used is: bp, base pair(s).
producing the SHELex26(26A)+26 coding sequence. The constructs of SHELex26A(P534A), SHELex18(26A), SHELex26A, and SHELex(26,26A)+26 were verified by DNA sequencing, and the proteins were prepared following the procedures used for SHELex26A.

SHELex17–27 is a 25.5-kDa polypeptide corresponding to domains 17–27 of SHEL26A (Fig. 1) and was obtained by overexpression from the plasmid pSHEL17–27 in E. coli BL21(DE3) as described previously (19).

SHELex18, SHELex20, and SHELex24 correspond to constructs of isolated domains 18, 20, and 24, respectively, of human tropoelastin and were obtained by amplifying their respective sequences from the plasmid pSHEL26A. The amplification of exon 18 used the primers 5′-GGA TCC GGT GCG GCA GCA GG-3′ and 5′-GGA TCC GGT GCG GCA GCA GG-3′. SHELex26A(P534A) was produced by amplifying the required sequence using the same primers as those used in the construction of SHELex26A(P534A) and was produced by overexpression from SHEL17–27 of SHEL-P534A, which comprises the central region (25,5-kDa) of SHEL17–27. SHELex26(A) is a 25.5-kDa polypeptide corresponding to domains 18, 20, and 24. SHELex26(26A), which comprises the central region of human tropoelastin harboring hydrophobic domains 18, 20, and 26. SHELex17–27 displayed CD profiles similar to those reported for the isolated hydrophobic domains, including an
increase in structure with increasing temperature. Coacervation of SHEL17–27 began only when the temperature reached 60 °C (Fig. 4a), and maximal coacervation was achieved at 65–70 °C (Fig. 4b). This was in contrast with the isolated hydrophobic domains that did not coacervate.

Results from previous experiments proposed a nucleation-condensation model for coacervation of full-length tropoelastin SHEL26A at 37 °C (21). This model, together with the putative dominant role for domains 18, 20, 24, and 26 during coacervation, suggested that even though SHEL17–27 did not coacervate at 37 °C, it may have been able to self-polymerize as part of the nucleation phase prior to coacervation. Sedimentation equilibrium studies at 37 °C showed that SHEL17–27 was soluble and existed predominantly in monomer form (data not shown). A monomer-dimer model also fitted the sedimentation data and returned a very weak association constant of ~600 M⁻¹, in which monomer represented at least 97% of the total mass at the highest protein concentration (~5.4 g/liter). This result did not provide evidence for a monomer-dimer association but suggested that SHEL17–27 may be able to form oligomers at 37 °C at substantially higher concentrations.

Properties of Tropoelastin Mutants—Four mutant forms of tropoelastin, SHELΔ26A(P534A), SHELΔ(18,26A), SHELΔ(26,26A), and SHELΔ(26,26A)+26, were analyzed for their secondary structure and ability to coacervate compared with SHEL26A. CD spectroscopy revealed that all four mutants had CD spectra similar to those of SHEL and SHELΔ26A (6, 22). These spectra have an intense negative minimum at 200 nm and a shoulder near 220–225 nm (Fig. 5). The minimum at 200 nm is typical of a largely unordered polypeptide, although a polypeptide rich in β-structure may exhibit a similar spectrum (23). Analysis of the CD spectra predicted that their secondary structure contents were dominated by contributions from β-structures (Table 1). Noticeably, β-turns represented 18% of the overall secondary structure contents in SHELΔ26A(P534A) compared with ~30% in other tropoelastin constructs. An increase in temperature from 10 to 70 °C caused a linear and gradual increase in structural content as measured at 200 nm for all tropoelastin forms (data not shown).

These four tropoelastin mutants were tested for their ability to coacervate relative to SHELΔ26A under the same conditions. These mutants are soluble at room temperature and coacervate reversibly when the temperature is raised. As seen for SHEL and SHELΔ26A, each mutant coacervated more rapidly at higher temperatures (Fig. 6c) and more rapidly at higher concentrations (Fig. 6b). Their coacervation abilities at 5 g/liter were compared with that of SHELΔ26A (Fig. 6c). Because these tropoelastin forms are of comparable sizes, their molar concentrations were similar. Table II lists the temperatures for each tropoelastin form to achieve 50 and 100% coacervation. SHELΔ26A completed 50 and 100% coacervation at 28 and 35–37 °C, respectively, whereas the mutant constructs required higher temperatures to achieve the same levels of coacervation. It is apparent that those disruptions involving deletion, rearrangement, or a single amino acid mutation involving the hydrophobic domain(s) of tropoelastin had deleterious effects on coacervation. The tropoelastin forms tested here were ranked on the basis of their coacervation ability from the most efficient to the least efficient as follows: SHELΔ26A, SHELΔ26A(P534A), SHELΔ(18,26A), SHELΔ(26,26A)+26, and SHELΔ(26,26A).

### DISCUSSION

The aim of the work presented here was to examine the contextual roles of individual hydrophobic domains in the intact tropoelastin molecule. The CD spectra of individual hydrophobic domains SHELΔ18, SHELΔ20, and SHELΔ24 and a mutant form of domain 26, SHELΔ(26,26A), displayed structural characteristics similar to those reported for the native domain 26, SHELΔ26 (15). Trifluoroethanol and, to a smaller extent, an increase in temperature induced conformational changes in all peptides, mainly in their β-content. While the structural transition in SHELΔ26 appeared to reach a plateau at 37 °C (15), none of the peptides described here showed the same effect. Therefore, the results from CD spectroscopy studies pointed to a lesser role in detecting the approach to 37 °C played by domains 18, 20, and 24 compared with domain 26. Furthermore, the Pro → Ala mutation in domain 26 caused structural and functional impairment as seen in SHELΔ26(P534A).

SHEL17–27 represents the central region of tropoelastin with the four largest Pro-Gly-containing hydrophobic domains, 18, 20, 24, and 26. SHEL17–27 coacervated at high temperatures and showed a propensity to self-associate at 37 °C. These results indicated a greater capacity for intermolecular interactions by SHEL17–27 compared with individual hydrophobic domains. It is possible that at a sufficiently high concentration, SHEL17–27 could form a high molecular weight oligomer that may be representative of the nucleating species for the nucleation-condensation model described for tropoelastin (21). These oligomers, however, may not necessarily be dimers and do not account for the coacervation of full-length tropoelastin under physiologically relevant conditions at 37 °C. Our experiments centered on the effect of mutations in the key hydrophobic domains on the coacervation of the full-length molecules. Although these mutants did not represent naturally synthesized tropoelastin splice variants, they allowed direct observations of the possible functions of the individual domains. Secondary structure analyses using CD spectroscopy showed that the four tropoelastin mutants, SHELΔ26A(P534A), SHELΔ(18,26A), SHELΔ(26,26A), and SHELΔ(26,26A)+26, were comparable with the native SHELΔ26A (22). The CD spectra reported here are similar to those described for other elastin peptides, including bovine tropoelastin, α-elastin, κ-elastin, and chemically synthesized elastin polypeptides (24–27). Overall, the tropoelastin constructs, regardless of their sizes or mutations, constituted very little α-helix and showed around 50% β-content. A reduction in the β-turn content in SHELΔ26A(P534A) compared with other tropoelastin constructs may suggest the disruption in the formation of type II β-turns in domain 26. Under more physiologically relevant conditions (such as at 37 °C, in 10 mM sodium...
phosphate, pH 7.4, and 150 mM NaCl), the tropoelastin constructs could assume distinct structural conformations that would be indicative of their coacervation ability. However, it was not possible to undertake CD studies under these conditions because the tropoelastin constructs would coacervate and chloride ions absorb strongly below 200 nm. Nevertheless, it appeared that disruptions to the hydrophobic domains did not have any significant effect on their overall secondary structure content in a normal aqueous environment.

Coacervation experiments showed that the four mutants coacervated endothermically and reversibly in a manner similar to that reported for tropoelastin isoforms SHEL and SHELΔ26A (6, 18). Increased protein concentrations sharpened the coacervation profiles and lowered the temperatures required for both the onset and the completion of coacervation, indicating the cooperative nature of their association. Despite the similarities in secondary structure content and in their coacervation profiles, it was clear that disruptions of the hydrophobic domains were detrimental to coacervation. These mutants required temperatures much higher than the normal physiological temperature to achieve full coacervation. Also, they displayed variable degrees of coacervation ability as judged by their midpoint coacervation temperatures.

Domain 18 of tropoelastin is also referred to as the W4 sequence. This sequence in bovine or porcine tropoelastin is composed of 11 regular VPGVG repeats, whereas the human counterpart contains nine irregular VPGVG-like pentameric repeats. Yet the polymer of human W4 sequence was functionally and structurally equivalent to poly(VPGVG) and the polymers of bovine and porcine W4 (28). Thus, this domain is likely to contribute to hydrophobic folding, possibly through the formation of type II β-turns and a β-spiral during coacervation (25, 29). This explains why the deletion of domain 18 in SHELΔ(18,26A) increased the midpoint coacervation temperature by 8 °C compared with SHELΔ26A.

Domain 26 of tropoelastin consists of four nonapeptide repeats of the sequence XGXXGXGVPG, where X is either A, L, V, or I. It plays a dominant role in coacervation (15). The complete removal of domain 26 in SHELΔ(26,26A) raised the midpoint coacervation temperature by 11 °C compared with SHELΔ26A. Evidently, the impact of the deletion of domain 26 in SHELΔ(26,26A) was more pronounced than the deletion of domain 18 in SHELΔ(18,26A), possibly because of the position of domain 26 on the surface of the molecule (15) and its involvement in intermolecular interactions. In SHELΔ26A(P534A), the Pro→Ala mutation is in the second nonapeptide of domain 26, where it would be expected to interfere with the formation of a type II β-turn during coacervation. It is significant that a single amino acid mutation in the 699-residue polypeptide resulted in a higher midpoint coacervation temperature than that seen for native SHELΔ26A. This finding is consistent with the dominant role played by domain 26 (15).

When domain 26 was placed at the C-terminal end of the molecule in SHELΔ(26,26A)+26, the midpoint coacervation temperature increased by 9 °C relative to that of SHELΔ26A. In comparison with the complete removal of domain 26 in SHELΔ(26,26A), the coacervation ability was improved; but it was not restored to the same level as that of native SHELΔ26A simply by the reinstatement of domain 26 at the C terminus. As shown here, both SHELΔ26A and SHELΔ(26,26A)+26 contain the same numbers of hydrophobic domains; yet the coacervation ability of SHELΔ(26,26A)+26 was significantly impaired because of the displacement of domain 26. This result argues for a contextual contribution of domain 26 in coacervation.

One factor that determines a contextual capacity for intermolecular association is the number of hydrophobic domains. As shown here, the coacervation ability was lessened when the
molecule lacked domains 18 and 26 (Fig. 7, a and d). By analogy, SHEL17–27, which contains four hydrophobic domains, coacervated at temperatures above 60 °C and showed a tendency to self-associate at 37 °C, whereas the individual hydrophobic domains remained completely monomeric. More significantly, the coacervation ability was also determined by the composition and position of these hydrophobic domains. Sequence mutation and/or rearrangements of these hydrophobic domains would adversely affect this ability even though the overall hydrophobicity was maintained (Fig. 7, a–c). The results support the important role of domain 26 and its precise location in the molecule. Furthermore, they demonstrate that coacervation very likely requires specific alignment of hydrophobic domains, which would also be a prerequisite for the juxtaposition of cross-linking sequences.

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FIG. 7. Diagrammatic representation of coacervation of tropoelastin constructs. a, intact tropoelastin such as SHEL and SHELΔ26A. b, tropoelastin containing a mutation in a hydrophobic domain such as SHELΔ26A(P534A). c, tropoelastin with a displaced hydrophobic domain such as SHELΔ(26,26A)+26. d, tropoelastin lacking a hydrophobic domain such as SHELΔ(18,26A) and SHELΔ(26,26A). The structural elements are defined at the bottom of the figure.
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