Glycosaminoglycans Mediate the Coacervation of Human Tropoelastin through Dominant Charge Interactions Involving Lysine Side Chains*

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Wendy J. Wu§, Bernadette Vrhovski‡, and Anthony S. Weiss¶

Department of Biochemistry, University of Sydney, Sydney, NSW 2006, Australia

Following cellular secretion into the extracellular matrix, tropoelastin is transported, deposited, and cross-linked to make elastin. Assembly by coacervation was examined for an isoform of tropoelastin that lacks the hydrophilic domain encoded by exon 26A. It is equivalent to a naturally secreted form of tropoelastin and shows similar coacervation performance to its partner containing 26A, thereby generalizing the concept that splice form variants are able to coacervate under comparable conditions. This is optimal under physiological conditions of temperature, salt concentration, and pH. The proteins were examined for their ability to interact with extracellular matrix glycosaminoglycans. These negatively charged molecules interacted with positively charged lysine residues and promoted coacervation of tropoelastin in a temperature- and concentration-dependent manner. A testable model for elastin-glycosaminoglycan interactions is proposed, where tropoelastin deposition during elastogenesis is encouraged by local exposure to matrix glycosaminoglycans. Unmodified proteins are retained at ~3 μM dissociation constant. Following lysyl oxidase modification of tropoelastin lysine residues, they are released from glycosaminoglycan interactions, thereby permitting those residues to contribute to elastin cross-links.

Human tropoelastin is the soluble monomer of elastin, which is largely responsible for the elastic properties of human lungs, large blood vessels and skin. Interactions of human tropoelastin with proteins such as microfibril-associated glycoprotein (MAGP-1) and 67-kDa elastin-binding protein (EBP) have been described (1–4). The assembly of the elastin fiber is also affected by glycosaminoglycans (GAGs) which may participate in the regulation of elastin synthesis and deposition during periods of new elastogenesis (5, 6). These GAGs are the substantially major components of the relevant proteoglycans. In this context, appreciable amounts of GAGs have been found in close association with the elastic fiber, prompting the view that these molecules could serve to modulate elastic fiber function (7). Using an elastin derivative, Velebný et al. (8) suggested that tropoelastin aggregates upon exposure to chondroitin sulfate. Heparin and heparan sulfate protect elastin from proteolytic attack by human leukocyte elastase (9) and stimulate elastogenesis (10). The saccharide units of dermatan and chondroitin sulfates differ from those of heparin-based GAGs because of their galactosamine content, and they display differing negative charge densities. The addition of these GAGs to a culture medium can differentially influence the deposition of elastin in the extracellular matrix (6). The molecular basis of these interactions is obscure although it is appreciated that GAGs serve to modulate the physical properties of the extracellular matrix by swelling in the presence of water, so bestowing diverse effects including electromechanical and physico-mechanical properties, lubrication, impact absorption, and molecular sieving, essentially as proteoglycan components (10, 11). Local concentrations of GAGs and their distribution can have profound effects on tissue remodeling and elastin fiber assembly (12). Chondroitin sulfates are closely associated with the developing elastic fiber (13) where these GAGs occur at sites of elastin deposition in the healing of full-thickness wounds (14) and may be closely associated with the elastic fiber (15). EBP is believed to accompany tropoelastin to the nascent elastic fiber, at which interactions with endogenous galactoside-containing GAGs mediate release of bound tropoelastin (16). At this point, tropoelastin is likely to encounter these molecules in the extracellular matrix. This warrants a study of the binding of GAGs to human tropoelastin. We show here that GAGs can provide a significant role in elastogenesis by promoting the association of tropoelastin molecules by coacervation.

EXPERIMENTAL PROCEDURES

Production and Expression of Tropoelastin Lacking 26A (SHELΔ26A)—Tropoelastin containing 26A (SHEL) was obtained as described previously (17). The SHELΔ26A gene was constructed from pSHELF by site-directed mutagenesis (Transformer Mutagenesis Kit, CLONTECH) using the oligonucleotide 5'-CGG GTT TCG GTG CTG TTT CCG CGG CGC TGG-3' that flanked either side of exon 26A by 15 base pairs, resulting in its precise deletion. This was followed by subcloning to provide the final construct in pET3d, which was confirmed by DNA sequencing. For SHEL and SHELΔ26A, full-length protein was obtained (17) and purified further by C18 reverse phase high performance liquid chromatography using a linear gradient of 20–80% (v/v) acetonitrile, 0.1% (v/v) trifluoroacetic acid. The masses of SHEL and SHELΔ26A were estimated, respectively, by electrospray mass spectrometry as 63,495 and 60,026, which compare well with the calculated masses of 63,489 and 60,017. Tropoelastin for Figs. 1 and 2 was obtained using protein from shaker flasks, and subsequent experiments used protein from cells grown in a BioFlo III fermentor where final purification was on a Waters PrepLC C18 reverse phase high performance liquid chromatography coupled with a Millennium-driven Waters 600 controller and pump.

Standard Binding Assay—An ultrafiltration assay was developed to examine interactions between heparin and two forms of tropoelastin,
SHEL and SHELΔ26A. [N-sulfonate-35S]heparin (Amersham Pharmacia Biotech; 1 μl) was incubated with 200 μl of 50 mM to 10 μM protein in phosphate-buffered saline (PBS) overnight at 4 °C to model buffer and salt conditions in the extracellular matrix (18). The final concentration of heparin was ~1.6 μl. In each case after mixing, a 150-μl aliquot was transferred to a centrifugal filter (Millipore Ultrafree-MC; 30,000 nominal molecular weight limit) and spun for 10 min at 4 °C. This was washed with 150 μl of PBS. 5 μl of filtrand samples from before and after washing were transferred to 1 ml of ACSII liquid scintillation fluid (Amersham Pharmacia Biotech) for counting using a Wallac (Finland) 1409 System 1400 liquid scintillation counter.

RESULTS

Tropoelastin Lacking Domain 26A (SHELΔ26A) Shows Coacervation Performance Similar to That of Tropoelastin (SHEL)—SHELΔ26A was compared with SHEL for its ability to reversibly associate by coacervation (18). This feature served to compare protein association behavior of the two forms of the protein and provided the basis for subsequent studies. SHEL (17) and SHELΔ26A were expressed in Escherichia coli to provide polypeptide that was identical to that inferred from cDNA sequences that had been isolated from human fetal aorta (19). These structural proteins are capable of thermodynamically reversible association by coacervation (e.g. 18, 20). SHELΔ26A was found to display similar coacervation characteristics to SHEL at comparable concentration, NaCl concentration, and pH. Thus, an increase in the concentrations of SHELΔ26A and NaCl substantially decreased the temperature of coacervation. An increase in pH more subtly also decreased the temperature of coacervation (Fig. 1; Ref. 18). At similar protein concentrations, repeated studies revealed that the temperatures of coacervation were slightly lower for SHELΔ26A than for SHEL and tended toward concentration independence at just below 37 °C (Fig. 2). The overall characteristics of both proteins showed the same trends. When all the variables are superimposed, coacervation of both tropoelastin forms (>20 mg/ml) at pH 7–8 and 150 mM NaCl is essentially complete at 37 °C. These in vitro conditions are similar to those found in vivo in the extracellular matrix (18). Circular dichroism of SHELΔ26A and SHEL revealed similar profiles indicative of comparable folding (results not shown; Ref. 18). These assays demonstrated that the two splice forms of tropoelastin could be compared in heparin binding studies.

SHEL and SHELΔ26A Show Similar Heparin Binding Affinities—To test for the ability of GAGs to bind tropoelastin, radiolabeled heparin was mixed with protein then separated by ultrafiltration. Binding of heparin to SHEL and SHELΔ26A revealed indistinguishable responses (Fig. 3A), with half-maximal binding in the low micromolar range. Binding by these proteins accounted for ~50% radiolabeled heparin; this is explained by heterogeneity commonly observed in commercial sources of heparin (21). Accordingly, binding of heparin to SHEL and to SHELΔ26A were each quantified using Scatchard analyses according to the method of Lee and Lander (21), where it was assumed that the concentration of GAG was much less than the $K_d$, the GAGs were not multivalent, and multiple high affinity sites in each tropoelastin form bound with similar $K_d$ values. Considering these assumptions, the $K_d$ values for the interaction between heparin and SHEL and with SHELΔ26A were estimated to be 2.5 and 4.5 μM, respectively (Fig. 3B). These comparable values provided an average $K_d$ ~3 μM. When isolated domain 26A was assayed as a fusion with glutathione S-transferase (GST26A), it showed a propensity to bind radiolabeled heparin. However, this association was weaker than the interaction of tropoelastin with heparin (results not
shown). This implicated a dominant participation by the most abundant positively charged amino acids (there are 35 lysines per molecule; Refs. 18 and 19) in tropoelastin.

GAGs Chondroitin Sulfate B and Heparin Increase the Propensity of Tropoelastin to Form Coacervates—Chondroitin sulfate B (1 mg/ml to 1 mg/ml) added to SHEL or SHELΔ26A significantly lowered the coacervation temperature. A similar effect was seen for both isoforms. Using tropoelastin (2 mg/ml), the addition of 1 mg/ml chondroitin sulfate B dramatically reduced the temperature required for coacervation, whereas as little as 5 μg/ml noticeably lowered the temperature for this process (Fig. 4, A and B). Thus the presence of this GAG can lower the coacervation of tropoelastin to below physiological temperatures and at higher concentrations to below room temperature.

The effect of heparin, while effective in the same concentration ranges as those for chondroitin sulfate B, clearly displayed a more substantial effect on coacervation at lower temperatures (Fig. 4, C and D). This indicated an aggregation of tropoelastin molecules prior to reaching the transition temperature because of multiple intermolecular interactions.

Glycosaminoglycan Interactions Are Mainly Mediated through Lysine Residues—Interaction with negatively charged GAGs was probably a consequence of the abundance of positively charged residues in tropoelastin, which contribute to a high, calculated isoelectric point of 11.2. The net positive charge status of tropoelastin drops in vivo upon cross-linking because of substantial lysine modification and participation in the production of oxidized and condensed lysine derivatives that are essential to the formation of elastin.

In the presence of chondroitin sulfate B, the coacervation curves for both forms of tropoelastin were displaced to lower...
temperatures by about 20–25 °C (Fig. 4, A and B). Heparin similarly displaced coacervation to lower temperatures, but in doing so it also flattened the curves, probably as a consequence of its different charge density and distribution compared with chondroitin sulfate B (Fig. 4, C and D). These effects were concentration-dependent to as little as 5 μg/ml GAG, whereas protein concentrations were considerably higher at 2–10 mg/ml. The GAG effects assumed that charge interactions played an important role in assisting aggregation of tropoelastin molecules. To examine the involvement of lysine side-chains in GAG interactions, the two forms of tropoelastin were treated with sulfosuccinimidyl acetate, which predominantly acetylates the epsilon amino group and converts it to charge neutrality (22). In the absence of GAGs, blocking of lysine only slightly (∼3–5 °C) shifted the coacervation curves from those of unmodified protein (Fig. 5). This indicates that positively charged, unmodified lysine residues are not major determinants of the temperature of transition of tropoelastin, probably because they are outside the directly associating hydrophobic regions (23). In contrast, chemical modification of the amino groups on lysine side chains showed a substantial effect on the influence of GAGs on coacervation. When the positive charges on lysine side chains were blocked, the effects of chondroitin sulfate B and heparin on coacervation were essentially abolished.

**DISCUSSION**

Tropoelastin assembly by coacervation is dramatically facilitated in the presence of the GAGs studied here. These effects are in the physiologically relevant range. For example, heparin binds to type I collagen with high affinity (K_d ∼ 150 nM) and triple helical peptides including the basic N-terminal sequence KGHRGF with intermediate affinities (K_d ∼ 2 μM). Thus heparin-type I collagen binding probably relies on an N-terminal basic triple-helical domain represented once within each monomer, and at multiple sites within fibrils (24). Similarly, human matrix metalloproteinase-2 degrades elastin in the ECM, where its C-domain binds heparin in the micromolar range (25). The two human tropoelastin isoforms used, SHEL and SHELΔ26A, differ by the presence or absence of 26A which contains the most hydrophilic repeat sequence in human tropoelastin. However, the two isoforms show similar coacervation properties at comparable protein concentrations, in the presence or absence of glycosaminoglycans. Therefore, the presence

![Fig. 4. Effect of glycosaminoglycans on coacervation of SHEL and SHELΔ26A.](image-url)
of this 26A region does not substantially affect the coacervation ability of SHEL despite increasing the hydrophilicity of this part of the protein. The coacervation data extend our earlier observations (18) with SHEL to a counterpart of a further splice form. Bedell-Hogan et al. (26) found that the isoform without 26A is a poorer substrate for lysyl oxidase and is not as readily cross-linked. The results shown here indicate that this difference is not caused by a difference in the ability of the isoforms to coacervate under these conditions.

The similar coacervation behavior of these two splice forms indicates that this feature is likely to be a general property of tropoelastin splice variants, where the transition temperature is finely tuned to occur under extracellular physiological conditions. The heparin binding studies were also conducted under these conditions. There is little evidence for the role of 26A (formerly referred to as 10A; Ref. 19) in tropoelastin and consequently for its physiological significance. This region alters lysyl oxidase reactivity on tropoelastin containing the sequence (26), and isolated 26A displays chemotactic activity for monocytes (27). While 26A is evident in human cDNA, homologous counterparts have yet to be identified in other animals. When subjected to Chou-Fasman and Garnier PepPlot (Genetics Computer Group, Wisconsin, Version 8) analyses, the peptide sequence shows no obvious propensity to fold and, indeed, predicts multiple alpha- and beta-breaking segments. In confirmation of this, we have subjected GST26A to x-ray crystallography, performed proton NMR solution structure studies on the isolated 26A region, and 26A circular dichroism analyses. Our data demonstrate that 26A is essentially random coil or highly flexible in aqueous solution. Apart from a reported type II $\beta$ turn with a 4-amino acid portion in high concentrations of trifluoroethanol, this is consistent with the observations of Bisaccia et al. (27). There appears to be greater heparin binding by GST26A than GST, suggesting a contribution by 26A to GAG association (data not shown) that might be relevant in the elastic fiber after elastogenesis.

Heparin binding by either tropoelastin isoform shows dissociation constants in the low micromolar range. This compares with the binding displayed by heparin to triple-helical peptide

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$^2$ Wilce, M., Wilce, J., Wu, W. J., and Weiss, A. S., unpublished data.
domains in type I collagen (24). Selective modification of lysine residues in tropoelastin should indicate the key contributors to this process. It is possible that the effects on coacervation are a consequence of the cumulative participation of an ensemble of lysines. The efficacy of low concentrations of GAGs suggests that interactions are effective with a subset of lysines. Different curve shapes for coacervation suggest that the effects of heparin and chondroitin sulfate B may be because of variations in negative charge densities in these GAGs.

The effect on coacervation would be less dramatic if lysyl oxidase converts most of the lysines in tropoelastin to the adipic semi-aldehyde, prior to significant exposure to GAGs. EBP (16) would assist in mediating correct delivery of tropoelastin to the nascent elastic fiber while minimizing inappropriate association with GAGs prior to polymerization to form elastin. GAG interactions occur in the extracellular matrix, which contributes to tropoelastin aggregation in its appropriate environment outside the cell. We propose that tropoelastin is encouraged to participate in elastogenesis by association with GAGs. As a consequence of this interaction, some of these GAGs may become entrapped in the nascent elastic fiber, as observed microscopically by Fornieri et al. (5). This displays an elegant dual role for GAGs: assisting in the release of tropoelastin from its partner EBP (16), then subsequently promoting coacervative formation and elastin deposition. In this way, GAGs serve to actively mediate tropoelastin interactions and encourage elastogenesis.

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