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Tropoelastin binding to fibulins, nidogen-2 and other extracellular matrix proteins

Takako Sasaki[a], Walter Göhring[a], Nicolai Miosge[b], William R. Abrams[c], Joel Rosenbloom[c], Rupert Timpl[a,*]

[a] *Max-Planck-Institut für Biochemie, Am Klopferspitz 18A, D-82152 Martinsried, Germany*
[b] *Center of Anatomy, Dept. Histology, University of Göttingen, D-37075 Göttingen, Germany*
[c] *Dept. Anatomy and Histology, School of Dental Medicine, University of Pennsylvania, Philadelphia, PA 19104, USA*

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Abstract Elastic fibers in vessel walls and other tissues consist of cross-linked tropoelastin in association with several microfibrillar proteins. In order to understand the molecular basis of these structures, we examined the binding of recombinant human tropoelastin to other extracellular matrix ligands in solid phase binding and surface plasmon resonance assays. These studies demonstrated a particularly high affinity (K_d about 1 nM) of tropoelastin for microfibrillar fibulin-2 and the recently described nidogen-2 isoform. More moderate affinities were observed for fibulin-1, laminin-1 and perlecan, while several other ligands such as collagens, nidogen-1, fibronectin and BM-40 showed little or no binding. In immunogold staining of mouse aortic media, elastic fibers were heavily decorated with tropoelastin, fibulin-2 and nidogen-2, while the reaction with fibulin-1 was lower. The colocalization of these proteins emphasizes the potential for in vivo interactions.

Key words: Elastic fiber; Immunogold staining; Protein interaction; Recombinant protein

1. Introduction

Elastic fibers are characteristic extracellular structures found in aorta and other vessel walls, lung, skin, elastic ligaments and cartilages, where they play a major role in allowing the reversible deformation of tissues [1–3]. The core structure of elastic fibers is composed of tropoelastin (72 kDa), which forms a highly insoluble network through extensive cross-linking mediated by lysyl oxidases. Four different models have been proposed for the structure of these networks in order to explain the mechanism of elasticity [1,2]. Due to its unique sequence, tropoelastin is also known as the most hydrophobic protein present in the extracellular matrix of vertebrates.

The supramolecular organization of elastic fibers also includes abundant 10–12 nm microfibrils which are often deposited at the periphery and considered to provide a scaffold for elastogenesis [1–3]. Major components of these microfibrils are the 350 kDa proteins fibrillin-1 and fibrillin-2 and natural mutations of both fibrillins are known to cause Marfan syndrome and related inherited disorders [4–6]. Further components include the latent transforming growth factor-β binding proteins (LTBPs), which are the closest structural relatives of the fibrillins [7]. As shown mainly by ultrastructural colocalization studies, many more constituents are likely to be involved in these microfibrillar arrays. Potential candidates are the 115 kDa emilin [8,9], the small microfibril-associated glycoproteins MAGP-1 and MAGP-2 [10,11], as well as several other designated microfibril-associated proteins (MFAP) [12–14], fibulin-1 [15] and fibulin-2 [16], lysyl oxidase [1,2], proteoglycans [3,17] and the angiogenesis inhibitor endostatin [17].

How these various constituents interact with one another at the molecular level is much less clear and only a few in vitro binding studies have been reported. They demonstrated binding of MAGP-1 to the C-terminal region of tropoelastin and no binding of fibrillin-1 [18,19]. Fibulin-2, however, shows a distinct binding affinity for an N-terminal segment of fibrillin-1 [16]. Likewise, endostatin may associate with the elastic fibers of vessel walls through binding to fibulin-2 and/or nidogen-2 [17].

Recombinant tropoelastin and several deletion mutants have now become available in sufficiently high quantities by production in bacteria [20–24]. This tropoelastin was shown to interact with cells [20,24] and lysyl oxidase [22] and to form coacervates in a temperature-dependent fashion [24]. In the present study, we have used recombinant tropoelastin in two different binding assays in order to identify potential extracellular matrix ligands. This demonstrated a particularly high affinity for fibulin-2 and nidogen-2 and more moderate interactions with fibulin-1, laminin-1 and perlecan. Several of these ligands could be localized by immunogold staining to the aortic media. This indicates a high complexity in the supramolecular organization of elastic fibers not previously recognized.

2. Materials and methods

2.1. Sources of proteins

Recombinant human tropoelastin which lacks the hydrophilic exon 26A was obtained by production in bacteria [20,22] and also used to prepare an antiserum. Laminin-1 complexed to nidogen-1, collagen IV and perlecan were purified from a mouse tumor basement membrane [25]. Mouse nidogen-1 [26], fibulin-1C [27], fibulin-2 [28], endostatin [29], human nidogen-2 [30] and BM-40 [31] were prepared in recombinant form from the culture medium of transfected human cells. Proteolytic fragments of laminin-1 [25] and recombinant fragments of fibulin-2 [32] and perlecan (see [33] for a summary) were obtained as previously described. Bovine serum albumin (BSA) (Serva) and human plasma fibronectin (Behringwerke) were commercial products.

2.2. Protein binding assays

Solid phase binding assays with one ligand immobilized onto the plastic surface of microtiter wells followed previously used methods [34]. Tropoelastin (2.67 mg/ml) was dissolved in 0.1% trifluoroacetic acid, 20% acetonitrile and stored at −70°C. After dilution to 10 μg/ml in 0.05 M Tris-HCl, pH 7.4, 0.15 M NaCl (TBS), it was used imme-

*Corresponding author. Fax: +49 (89) 8578 2422.
E-mail: timpl@biochem.mpg.de

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diately to coat the wells, followed by blocking with 1% BSA. Control wells were coated and blocked with BSA alone in order to determine non-specific binding. Soluble ligands (0.01 to 1000 nM) were dissolved in 1% BSA/TBS and their binding was detected by specific antisera [27,28,30].

Surface plasmon resonance assays were performed with BIAcore 1000 instrumentation (BIAcore AB). Tropoelastin was diluted to 200 μg/ml in 0.1 M sodium acetate pH 4.0 and covalently coupled [30] to CM-5 sensor chips (research grade) at 25°C for 12 min (flow rate 5 μl/min). Immobilization levels were adjusted to 3000–6000 resonance units (RU), which avoided mass transport problems and allowed sufficiently high binding signals (30–450 RU) for a precise evaluation. Binding assays were performed with ligands (5–500 nM) dissolved in TBS containing 0.05% P20 surfactant (BIAcore AB) and 2 mM $CaCl_2$ at 25°C and a flow rate of 20 μl/min. Initial experiments showed that this flow rate was high enough to eliminate mass transport problems. Associations were monitored for 3 min followed by a dissociation phase of 30 min. Uncoated chips or chips coupled with BSA were used to exclude non-specific binding and bulk effects. Kinetic constants were calculated by non-linear fitting of the association and dissociation curves according to a 1:1 model A+B = AB following the manufacturer's instructions (BIAevaluation software version 3.0).

2.3. Immunogold staining

Immunoelectron microscopy followed recently applied procedures [17,35]. Briefly, pieces of aorta (about 1 mm^2) from adult NMRI mice were fixed in 4% paraformaldehyde and 0.5% glutaraldehyde and used to obtain ultrathin sections. The labeling of 16 nm gold particles with affinity-purified goat anti-rabbit IgG (Medac) followed established protocols. Tissue sections were incubated with the antiserum against tropoelastin (1:300) or affinity-purified rabbit antibodies against fibulin-1 (6 μg/ml), fibulin-2 (17 μg/ml) or nidogen-2 (4 μg/ml) and subsequently with the gold-labeled antibodies (1:200). After staining with uranyl acetate and lead citrate, sections were examined with a Zeiss EM 109 electron microscope. Control sections that were incubated with the gold-labeled antibodies showed no staining.

3. Results

Solid phase binding assays were used as an initial screen for the binding of potential ligands to immobilized tropoelastin (Table 1). A particularly strong interaction was observed with fibulin-2 and binding levels were about 10-fold lower with nidogen-2 and the proteoglycan perlecan. The interactions

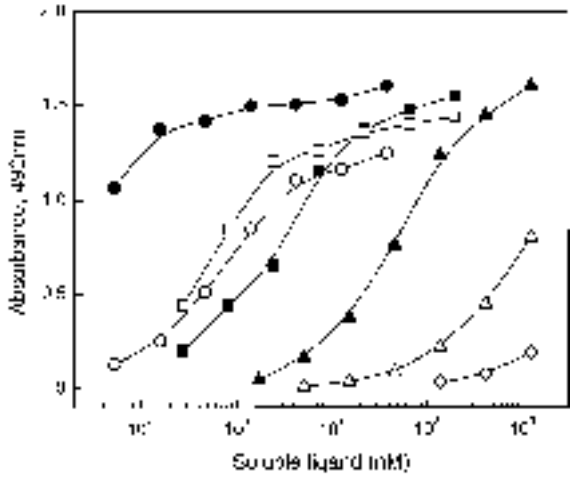

Fig. 1. Binding of fibulins and nidogen-2 to plastic-immobilized tropoelastin. Assays were carried out in the presence of 2 mM $CaCl_2$ (closed symbols) or 4 mM EDTA (open symbols). Soluble ligands were fibulin-2 (●, ○), nidogen-2 (■, □) and fibulin-1C (▲, △). Binding to serum albumin as a specificity control is only shown for fibulin-1C (◇).

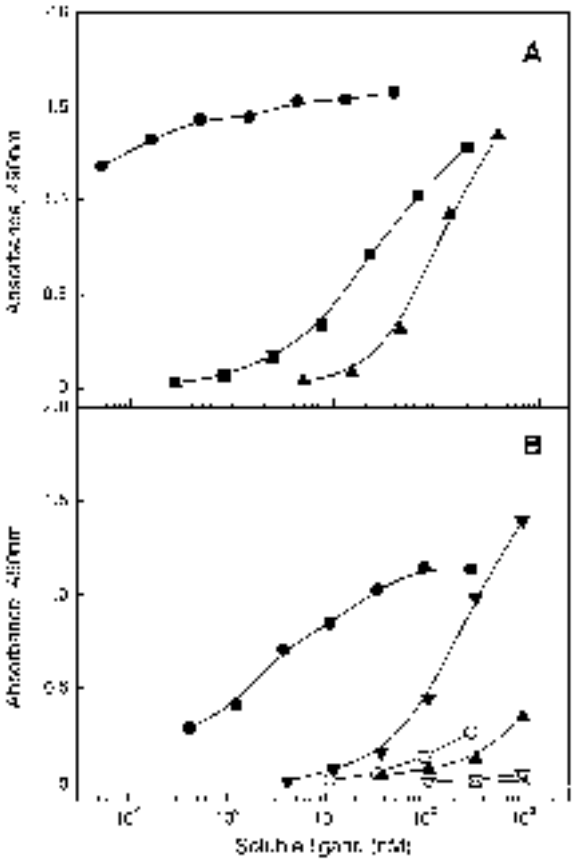

Fig. 2. Binding of recombinant fragments of fibulin-2 (A) and perlecan (B) to plastic-immobilized tropoelastin. Soluble ligands in (A) were fibulin-2 (●) and the fibulin-2 fragments N+I (■) and II+III (▲). Soluble ligands in (B) were perlecan (●) and the perlecan fragments III-2 (▼) and IA (▲). Control binding to serum albumin is shown by open symbols.

with fibulin-1C, laminin-1 and fibronectin were even weaker. Several other extracellular ligands (collagens IV and VI, endostatin, BM-40) were inactive up to a concentration of 300–1000 nM. This strongly indicated that tropoelastin discriminates between different protein ligands rather than binding non-specifically due to its hydrophobic character.

The interactions were dose dependent and several ligands showed a clear plateau in their binding profiles when used at

Table 1
Binding of soluble ligands to immobilized tropoelastin and serum albumin in solid phase assays

Soluble ligand	Tropoelastin		Serum albumin	
	(OD)	(nM)	(OD)	(nM)
Fibulin-1C	1.6	40	0.1	–
Fibulin-2	1.6	<0.05	0	–
Nidogen-1	1.8	40	1.0	>100
Nidogen-2	1.6	3	0.6	>200
Perlecan	1.2	3	0.2	–
Laminin-1	1.1	50	0.4	–
Fibronectin	1.3	80	0	–

Parameters of binding recorded are absorbance (OD) at 490 nm (either in the plateau region or at 300 nM) and the concentration required for half maximal binding (nM). In the assays with fibulins and nidogen-2 2 mM $CaCl_2$ was added to the buffer. Laminin-1 was used as a complex with nidogen-1.

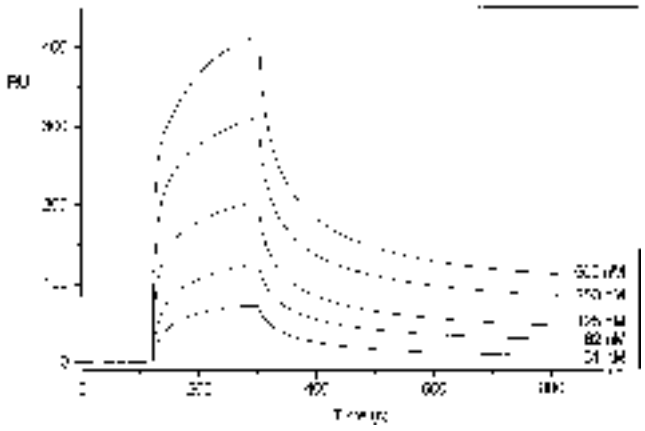

Fig. 3. Concentration dependence of the binding of fibulin-2 to immobilized tropoelastin in surface plasmon resonance assays. Soluble ligands were injected at 120 s and replaced by buffer at 300 s. Uptake and dissociation of protein is recorded in resonance units (RU).

high concentrations (Figs. 1 and 2). Furthermore, most of the ligands showed no significant binding to serum albumin (Table 1) which was used to block the wells coated with tropoelastin. The exception was nidogen-1, leaving it open as to whether the slight increase in binding to tropoelastin represents a specific interaction. Efficient binding of fibulin-1C and fibulin-2 to tropoelastin was dependent on calcium (Fig. 1), as shown previously for other fibulin ligands [27,28]. Addition of EDTA caused a 25- to 50-fold reduction in binding activity. This was, however, not the case for nidogen-2.

Surface plasmon resonance assays with immobilized tropoelastin were used to confirm and extend these binding data. Most of the identified ligands showed a typical concentration-dependent binding to tropoelastin, as illustrated for fibulin-2 in Fig. 3, which allowed association and dissociation rate constants to be determined (Table 2). The strongest affinity was again observed for fibulin-2 ($K_d = 0.6$ nM), while 20- to 30-fold lower affinities were found for fibulin-1C and laminin. No binding was observed with soluble nidogen-2, which could be due to destruction of binding epitopes on tropoelastin by its covalent coupling to the sensor chip through lysine residues. Immobilization of nidogen-2 showed a clear binding of soluble tropoelastin with $K_d = 3$ nM (Table 2), supporting this interpretation. No binding to tropoelastin was observed for nidogen-1, fibronectin and BM-40. Immobilized BSA was used as a control and showed no binding of fibulin-1C, fibulin-2, nidogen-2 and laminin-1.

Several tropoelastin ligands are also available in the form of larger proteolytic or recombinant fragments. These were now used for the mapping of binding epitopes. The N- and C-terminal fragments N+I and II+III, respectively, which together correspond to the entire fibulin-2 molecule [32], both showed only a weak binding to tropoelastin in solid phase assays (Fig. 2A) and no interaction was detected by surface plasmon resonance assay up to a concentration of 0.5 μM. However, a clear binding was observed for fragments E3 and E8 from the distal end of the long arm of laminin-1 [25], with an affinity comparable to that of the entire protein (Table 2). Other fragments (E1X, E4) from laminin's short arms showed no significant binding. A complete set of perlecan fragments [33] was also examined for tropoelastin binding by surface plasmon resonance assays. Only a single fragment, III-2, showed significant binding with $K_d = 21$ nM (Table 2). Since perlecan could not be used as the soluble ligand in this assay, a comparison was made in solid phase assays (Fig. 2B). This demonstrated distinct binding of tropoelastin to perlecan, at levels about 30-fold higher than to fragment III-2, and only little binding to perlecan fragment IA which contains the heparan sulfate chains.

Immunogold staining was used to examine whether elastic lamellae in the media of mouse aorta contain the potential ligands identified in the present study. Labeling was strong and at comparable levels for tropoelastin and fibulin-2, with moderate labeling for nidogen-2 and a low level for fibulin-1 (Fig. 4). Previous studies have shown a similar weak staining for perlecan [17] and no reaction with a monoclonal antibody against laminin-1 that reacts strongly with basement membranes in other tissues [35]. Most of the staining was on extracellular amorphous and microfibrillar regions. The antibodies to fibulin-2 and nidogen-2 also showed a distinct albeit less abundant intracellular localization, indicating either active synthesis or resorption of these proteins.

4. Discussion

The strong in situ association of amorphous elastic fibers with microfibrillar components has been known for a long time [1–3]. However, as yet only little information is available about specific molecular contacts from in vitro studies with purified proteins. By using such assays, the present study provides evidence that recombinant tropoelastin binds to fibulin-2, nidogen-2, perlecan, fibulin-1 and laminin-1. The affinity of binding decreased in the order of these ligands, as confirmed by two different assays. As shown here and previously [15–17] by ultrastructural analyses, all of these potential ligands ex-

Table 2
Surface plasmon resonance assays of tropoelastin binding to extracellular matrix ligands

Immobilized ligands	Soluble ligands	$k_d \times 10^3$ (s^{-1})	$k_a \times 10^{-3}$ (M^{-1} s^{-1})	K_d (nM)
Tropoelastin	fibulin-1C	2.5	135	18 ± 9
	fibulin-2	1.5	2470	0.6 ± 0.2
	nidogen-2		no binding	
	perlecan III-2	0.35	17	21
	laminin-1	0.75	71	11 ± 4
	laminin-1 E3	0.32	35	9 ± 4
	laminin-1 E8	1.4	86	16 ± 5
Nidogen-2	tropoelastin	0.85	306	3 ± 2

Soluble ligands were used at various concentrations (5–500 nM) and showed an uptake in the range 30–450 RU. Kinetic and thermodynamic constants are in most cases averages of 3–5 independent measurements with the error range (± S.D.) shown only for K_d values. Laminin-1 was complexed to nidogen-1. E3, E8 and III-2 refer to fragments of these proteins.

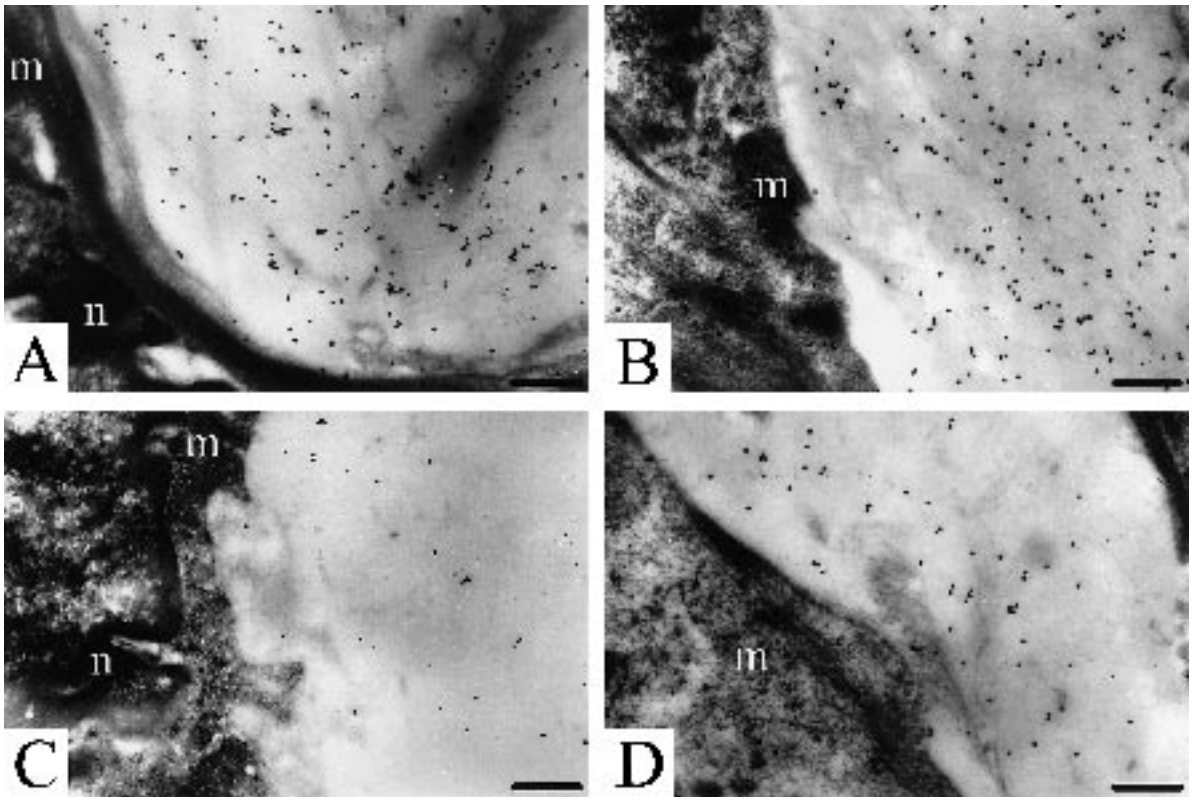

Fig. 4. Immunogold localization of tropoelastin and its potential ligands on elastic lamellae in the media of mouse aorta. Staining was with an antiserum against tropoelastin (A) and affinity-purified antibodies against fibulin-2 (B), fibulin-1C (C) and nidogen-2 (D). The major staining is on amorphous and microfibrillar parts of the matrix, but additional intracellular staining is also seen in (B) and (D). m, smooth muscle cell; n, nucleus. Bars indicate 0.25 μm.

cept laminin-1 colocalize on elastic tissue fibers. This makes it meaningful in a biological context to carry out a more precise investigation of the supramolecular organization of such specialized extracellular structures.

Fibulin-2 was by far the strongest ligand for tropoelastin. This protein consists of an elongated (70–80 nm) dimer with some flexibility in its structure [32] and was already known to be associated with microfibrils composed primarily of fibrillin or fibronectin [16,36,37]. This has been supported by direct binding of the proteins involved to one another [16,28] and additional fibulin-2 ligands such as nidogen-1, perlecan and endostatin were also identified in these assays [28,29]. Since tropoelastin seems not to have a distinct affinity for fibrillin [18], it is tempting to speculate that fibulin-2 could mediate the attachment of the corresponding microfibrils to elastin structures. The related component fibulin-1 has a 30-fold lower affinity for tropoelastin and does not bind to fibrillin [16], but does bind to several other extracellular ligands including laminin-1, nidogen-1 and endostatin [27,29]. The ultrastructural localization of fibulin-1 on skin elastic fibers indicates a primary interaction with the amorphous component rather than microfibrils [15], in agreement with the other observations. Our data on two rather low binding recombinant fragments of fibulin-2 that span the entire size of the protein [32] indicate that multiple interaction sites are required for efficient binding. They certainly include calcium binding domains II of both fibulin-2 and fibulin-1 since calcium seems to be essential for efficient binding to tropoelastin. This has set the stage for a more precise mapping of binding epitopes on fibulin-1, as shown in other studies for the ligands nidogen-1 [38] and the lectin binding domains of aggrecan and versican [39].

An unexpected but relatively strong ligand for tropoelastin was nidogen-2, which is an isoform of the ubiquitous basement membrane component nidogen-1. Nidogen-2 has a similar elongated (40–50 nm) structure and was localized to basement membranes and other extracellular regions [30] including, as shown here, the elastic lamellae of aorta. Nidogen-2 has a distinct in vitro binding capacity for perlecan, collagen IV and endostatin but its binding to laminin-1 is much lower [17,30]. Nidogen-1 is known to bind strongly to laminin-1 with $K_d = 0.5$ nM [26] but apparently has no distinct affinity for tropoelastin. Since the collagen XVIII-derived endostatin also has no affinity for tropoelastin, its localization to the aortic media may be explained by binding to either nidogen-2 or fibulin-2 [17].

Various elastic fibers are also known to contain heparan sulfate proteoglycans [3], including perlecan [17], the major proteoglycan of basement membranes [40]. Perlecan has a considerable potential for interactions with other extracellular ligands, many of which bind to the C-terminal domains IV and V or the N-terminal heparan sulfate chains [33]. Tropoelastin binding to perlecan, however, occurs with moderate affinity to the central domain III-2, which also carries a binding site for platelet-derived growth factor [41]. Laminin-1 is also a typical basement membrane protein and could not be detected on the elastic sheets of the aorta. Yet it showed a reasonable affinity for tropoelastin and we speculate that this binding may be involved in connecting elastic fibers to base-

ment membranes surrounding smooth muscle cells in vessel walls. The tropoelastin binding fragments E3 and E8 of laminin contain the five LG modules of the α1 chain which are implicated in major cellular interactions and possess strong binding epitopes for heparin and α-dystroglycan [42,43]. Whether tropoelastin modulates such activities remains an interesting aspect to study.

Elastin-associated proteins such as fibrillins, LTBPs, emilin and MAGP-1 [7,8,18,19,44] have been considered to be essential for elastogenesis in tissues and to promote the polymerization of tropoelastin. The five novel tropoelastin ligands described here may also be involved in these processes. The particular contribution of each component still needs to be clarified in order to fully understand the supramolecular organization of elastic fibers.

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