A Novel Cell Adhesion Region in Tropoelastin Mediates Attachment to Integrin $\alpha_V\beta_5^*$

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Background: Cellular integrin $\alpha_V\beta_5$ binds to RKKR at the C-terminal tail of tropoelastin.

Results: Inhibition of integrin $\alpha_V\beta_5$ hinders cell adhesion to tropoelastin constructs comprising domains 17 and 18.

Conclusion: Integrin $\alpha_V\beta_5$ mediates cell adhesion to tropoelastin through a central region.

Significance: Understanding the cell adhesion activity of tropoelastin gives improved insight into the physiological and pathological cell responses to this protein.

Tropoelastin protein monomers assemble to form elastin. Cellular integrin $\alpha_V\beta_5$ binds RKKR at the C-terminal tail of tropoelastin. We probed cell interactions with tropoelastin by deleting the RKKR sequence to identify other cell-binding interactions within tropoelastin. We found a novel human dermal fibroblast attachment and spreading site on tropoelastin that is located centrally in the molecule. Inhibition studies demonstrated that this cell adhesion was not mediated by either elastin-binding protein or glycosaminoglycans. Cell interactions were divergent cation-dependent, indicating integrin dependence. Function-blocking monoclonal antibodies revealed that $\alpha_V$ integrin(s) and integrin $\alpha_V\beta_5$ specifically were critical for cell adhesion to this part of tropoelastin. These data reveal a common $\alpha_V$ integrin-binding theme for tropoelastin: $\alpha_V\beta_5$ at the C-terminus and $\alpha_V\beta_5$ at the central region of tropoelastin. Each $\alpha_V$ region contributes to fibroblast attachment and spreading, but they differ in their effects on cytoskeletal assembly.

Cell interactions with the surrounding extracellular matrix (ECM)$^3$ are critical for a multitude of cell responses, including cell survival and tissue maintenance. Integrins are dominant cell surface receptors that help to mediate cell interactions with ECM components$^1$ that influence cell migration and proliferation, tissue organization, wound repair, development, and host immune responses. Integrins are heterodimeric transmembrane glycoprotein receptors comprising noncovalently linked $\alpha$- and $\beta$-subunits. In humans, at least 18 $\alpha$- and 8 $\beta$-subunits can dimerize to give diverse receptor combinations. This combination of $\alpha$- and $\beta$-subunits is the basis for ECM ligand specificity$^2$. It was once thought that integrins recognize only RGD (3); however, it is now appreciated that non-RGD motifs also serve as ligands. Additionally, many ECM proteins contain a distal domain that synergizes with the core attachment site to elicit full cell-binding activity and integrin receptor specificity. For example, fibronectin contains an upstream PHSRN synergy sequence that is required for full integrin-binding activity$^4$.

Integrin $\alpha_V\beta_5$ is widely expressed by cells such as fibroblasts, endothelial cells, and vascular smooth muscle cells, where it is involved in activation-dependent cell migration and cell adhesion to the ECM$^5$. Myofibroblasts express elevated levels of integrin $\alpha_V\beta_5$ during dermal wound repair$^6$. Alongside integrin $\alpha_V\beta_5$, integrin $\alpha_V\beta_5$ plays a major role in growth factor-induced angiogenesis through a distinct pathway; integrin $\alpha_V\beta_5$ acts through basic fibroblast growth factor and TNF-$\alpha$, whereas integrin $\alpha_V\beta_5$ acts through VEGF and TGF-$\alpha$(7). Integrins $\alpha_V\beta_3$ and $\alpha_V\beta_5$ interact with fibulin-5 and so may participate in elastogenesis through anchorage of elastic fibers to cells$^8$.

Elastic fibers are a key structural component of the ECM, where they provide elastic recoil and resilience to tissues that are subjected to repetitive extensile forces, including the skin, lungs, and vasculature. Elastin is involved in a range of interactions with fibroblasts, smooth muscle cells, and endothelial cells, where it influences chemotaxis, attachment, spreading, proliferation, and differentiation$^9$.

Tropoelastin and therefore elastin interact directly with cells through several cell surface receptors, including the elastin-binding protein (EBP)$^10$, glycosaminoglycans (GAGs)$^11$, and integrin $\alpha_V\beta_3$ $^12$. Tropoelastin can interact with cells indirectly via interactions with other ECM proteins such as fibulin-5$^8$(8, 13).

The 67-kDa EBP is a peripheral membrane splice variant of $\beta$-galactosidase that complexes with the integral membrane proteins carboxypeptidase A and sialidase to form a transmembrane elastin receptor$^14$. This receptor binds XGXXPG consensus sequences and, in particular, VGVAPG within exon 24 of elastin$^{15}$ Although binding elicits cell responses (16–20), it is not responsible for direct human dermal fibroblast (HDF)-tropoelastin interactions; rather, EBP appears to function as a detector of elastin-derived fragments that are generated when elastin is damaged$^{10}$. 

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3 The abbreviations used are: ECM, extracellular matrix; EBP, elastin-binding protein; GAG, glycosaminoglycan; HDF, human dermal fibroblast; HS, heparan sulfate; aa, amino acids.
Integrin-mediated Cell Adhesion to Tropoelastin

Cell surface heparan and chondroitin sulfate-containing GAGs serve as cell receptors for chondrocyte-tropoelastin interactions (11). This GAG-mediated cell-binding activity is located in the C-terminal 25 amino acid residues of bovine tropoelastin. Heparan sulfate (HS)-mediated interactions have also been noted for binding of human fibroblasts and smooth muscle cells to bovine tropoelastin (21). Although GAG-tropoelastin interactions are well established for bovine tropoelastin, this same interaction has not been shown for human tropoelastin. Presumably this is because this interaction occurs with sequences contributed by exons 34 and 35 of bovine tropoelastin that are absent in human tropoelastin (12).

As tropoelastin does not contain a classic integrin-binding RGD sequence, for some time, it was underexplored for potential integrin-binding ligands. Subsequently, integrin \( \alpha_\nu\beta_3 \) on HDFs was found to recognize the extreme C-terminal RKRK motif of human tropoelastin (12). However, this interaction does not account for the full cell-binding activity of tropoelastin. Human umbilical vein endothelial cells can attach to the N-terminal (encoded by exons 2–18), central (encoded by exons 18–27), and C-terminal (encoded by exons 18–36) domains of recombinant fragments of human tropoelastin (22, 23), although the mechanism of interaction is not known, and sequence non-specificity cannot be excluded. Here, we have used recombinant tropoelastin constructs to identify a new cell-binding site in tropoelastin and to identify integrin \( \alpha_\nu\beta_3 \) as the major receptor for this novel region.

EXPERIMENTAL PROCEDURES

Materials—Recombinant human tropoelastin was produced in-house (24). HDFs were sourced from the Coriell Research Institute (Camden, NJ). Anti-human integrin antibodies 17E6 (\( \alpha_\nu \)), P1D6 (\( \alpha_\nu\beta_3 \)), and P1F6 (\( \alpha_\nu\beta_3 \)) were from Abcam. All other reagents were from Sigma–Aldrich.

Tropoelastin Construct Expression—Tropoelastin constructs encoding ΔRKRK (aa 27–721), the N terminus to domain 25 (N25; aa 27–541), the N terminus to domain 18 (N18; aa 27–366), the N terminus to domain 10 (N10; aa 27–181), and domains 17–27 (17–27; aa 298–596) (see Fig. 1A) were cloned in-house into the pET3d vector from the WT full-length tropoelastin sequence corresponding to amino acid residues 27–724 of GenBank™ accession number AAC98394 (gi 182020). The constructs were cloned into Escherichia coli BL21(DE3) cells and overexpressed as described (24). The purified protein was further purified by reversed phase HPLC using an Agilent Technologies ZORBAX StableBond 300SB-C18 5-μm column. A gradient of 0–100% acetonitrile and 0.1% TFA over 1 h was used to elute protein fractions. SDS-PAGE (see Fig. 1B) confirmed single discrete species corresponding to predicted molecular masses.

Cell Culture—GM3348 HDFs were cultured in DMEM supplemented with 10% (v/v) FBS and passaged 1:3 every 3 or 4 days.

Cell Attachment and Spreading—Cell attachment and spreading analysis was performed as described (12). Tropoelastin-coated and denatured BSA-blocked wells were incubated with cell suspensions for 1 h at 37 °C. For attachment analysis, unless stated otherwise, the wells were washed three times with PBS to remove loosely adherent cells. For more stringent washing conditions, the cells were subjected to between one and seven sequential PBS washes before fixing the adherent cells in 3% formaldehyde. Adherent cells were stained with 0.1% (w/v) crystal violet in 0.2 M MES (pH 5.0) for 1 h. For cell spreading, cells were not aspirated before fixing in 3% formaldehyde solution. A cell suspension of 2.5 × 10^5 cells/ml was used for all attachment analysis, and a cell suspension of 1 × 10^5 cells/ml was used for all spreading analysis. For cell spreading assays, cells were visualized by phase-contrast microscopy with a Zeiss Axiovert 200M microscope at ×100 magnification, and photos were taken on a PixeLINK camera (model PL-A623) for cell spreading quantification. To observe the relative degree of cell spreading between tropoelastin constructs, the area of cell spreading was quantified using ImageJ. For inhibition studies, the degree of attachment was measured using a yes/no threshold methodology. Cells with a flattened phase-dark body and visible nucleus were considered spread, whereas cells that were rounded and phase-bright were considered unspread. The extent of cell spreading was read by three observers who were blinded to the sample identity. This method of quantification was shown to be comparable to calculation of cell area using ImageJ (data not shown).

Inhibition and Cation Add-back Studies—Inhibition studies were conducted as described above except that final concentrations of 5 mM EDTA, 10 mM β-lactose, 10 mM α-lactose, 10 mM d-glucose, 10 μg/ml HS, and 10 μg/ml antibodies 17E6, P1D6, and IgG were included during cell attachment or spreading. P1F6 was diluted 1:300 for cell attachment assays and diluted 1:150 for cell spreading assays.

To determine the effect of cations, the same methodology was used except that the cell pellet was suspended in cation-free PBS, centrifuged at 800 × g for 5 min, and resuspended in cation-free PBS. The cells were presented to the tropoelastin-coated surfaces with 0.05–0.4 mM cation at a final cell density of 2.5 × 10^5 cells/ml.

Immunofluorescence of Actin Cytoskeletal Assembly—Glass coverslips were placed into the wells of a 24-well tissue culture plate and incubated with tropoelastin constructs overnight at 4 °C. The tropoelastin solution was then aspirated, and any unbound surfaces were blocked with 1% (w/v) denatured BSA (80 °C/10 min) for 1 h at room temperature. Cells (500 μl) at a density of 2 × 10^5 cells/ml in serum-free DMEM were added to each well and incubated for 1.5 h at 37 °C. Cells were fixed with formaldehyde, and the actin cytoskeleton and nuclei were visualized as described (25).

Statistical Analysis—Experiments were performed in triplicate or quadruplicate as indicated, converted to mean ± S.D., and then analyzed using one- or two-way analysis of variance applied with Bonferroni post-tests. Data were accepted as statistically significant at \( p < 0.05 \).

RESULTS

The Tropoelastin C-terminal RKRK Motif Does Not Completely Account for Cell-binding Activity—In our previous study, although RKRK-containing peptides could support cell attachment, inhibition of C-terminal RKRK-dependent cell binding did not completely block the attachment of HDFs to...
WT tropoelastin (12). Although work showed that the C-terminal region bound integrin $\alpha_v\beta_3$, the relative importance of the region in the context of the rest of the molecule was not assessed. Therefore, to explore the role of RKRK in the context of the remainder of the tropoelastin molecule, it was removed from WT tropoelastin to generate the RKRK construct (Fig. 1A). HDFs bound to both WT and $\Delta$RKRK tropoelastin in a dose-dependent manner (Fig. 2A). Regardless of the concentration tested, cell binding to $\Delta$RKRK did not significantly differ from that to WT tropoelastin. Maximal cell attachment to $\Delta$RKRK (86.94 ± 2.92%) and WT (84.64 ± 1.53%) at 200 nM was indistinguishable. To further probe the cell-binding affinity of WT and $\Delta$RKRK, cell attachment assays were performed with increasing numbers of wash cycles to progressively remove more loosely adherent cells (Fig. 2B), which again showed comparable behavior. This suggested that another sequence(s) maintained tight cell binding elsewhere in tropoelastin.

To determine whether these constructs elicited differing signaling required for actin fiber assembly, cells bound to WT and $\Delta$RKRK were visualized by immunofluorescence confocal microscopy (Fig. 2C). WT and $\Delta$RKRK both supported cell attachment and spreading. However, cells on $\Delta$RKRK displayed an altered morphology and more cellular projections. Cells on $\Delta$RKRK presented markedly less actin fiber assembly than those on WT tropoelastin. Together, these data suggest that

![Schematic representation (A) and SDS-PAGE analysis (B) of tropoelastin-based constructs.](image-url)
although removal of the RKRK motif affects cell signaling, it does not alter the cell-binding affinity of tropoelastin.

Central Domains of Tropoelastin Support Cell Adhesion and Spreading—Constructs containing premature C-terminal truncations (the N terminus to domain 25 (N25), the N terminus to domain 18 (N18), and the N terminus to domain 10 (N10)) were used to delineate potential RKRK-independent cell-binding regions. The WT, N18, and N10 forms of tropoelastin showed dose-dependent adherence to HDFs (Fig. 3A). At maximal 200 nM coating concentrations, WT was more cell-adhesive (60.2 ± 4.9%) than N18 (51.2 ± 0.8%), which in turn was more cell-adhesive than N10 (22.2 ± 0.8%). N25 was used to assess whether domains C-terminally adjacent to N18 are required for full cell adhesion (Fig. 3B). At low coating concentrations, N25 presented slightly higher levels of cell-binding activity than N18. At concentrations at which cell attachment reached a plateau, cell adhesion to N18 (41.5 ± 2.8%) and N25 (42.2 ± 7.8%) did not differ, whereas both supported significantly less attachment than WT (54.6 ± 1.6%). Increasing the wash cycles for attached cells (Fig. 3C) showed comparable cell attachment for N18 and N25, suggesting that they both have the same cell-binding affinity. This is explained by a model in which domains 2–18 account for the cell-binding activity of this region, with no requirement for the C-terminal domains of tropoelastin.

The overlapping construct containing domains 17–27 (17–27) confirmed that the major cell-binding site in N18 is in domains 17 and 18 of tropoelastin (Fig. 4A). Both 17–27 and N18 supported dose-dependent cell attachment. At all concentrations, the attachment to 17–27 did not differ significantly compared with N18. Maximal cell attachment was comparable at 52.3 ± 2.8% and 47.0 ± 6.7% for 200 nM 17–27 and N18, respectively. Both 17–27 and N18 supported a lower level of cell attachment compared with WT tropoelastin (57.9 ± 4.4%). Increased wash cycles were used to further investigate the cell-
binding affinity between the constructs (Fig. 4B). With persistent washing (six to seven washes), the cell-binding activity was not statistically different between 17–27 and N18. In summary, these tropoelastin constructs showed clear differences in their ability to support HDF adhesion with affinities of WT/H11005/H9004/RKRK/H11022/N25/H11005/N18/H11350/17–27/H11022/N10.

Phase-contrast microscopy was used to visualize fibroblast spreading on 100 nM WT, H9004/RKRK, N18, 17–27 and N10 constructs after 60 min (Fig. 5A). N10 and the background control showed lower areas of cell spreading (495.1 ± 127.4 and 212.8 ± 17.2 μm, respectively) compared with WT (1085.4 ± 73.7 μm), ΔRKKR (939.4 ± 131.2 μm), N18 (967.4 ± 112.6 μm), and 17–27 (758.7 ± 73.4 μm). In addition, N10 supported larger cell areas compared with the background control. This was consistent with the trend seen in the previous cell attachment studies. The same trend was seen when cell incubation times were extended to 90 min (data not shown). Representative images (Fig. 5B) show that although N10 was slightly different from the background control, it did not support HDF spreading; the majority of cells were rounded and phase-bright. In contrast, on WT, ΔRKKR, N18, and 17–27, the majority of HDFs were phase-dark and had cellular projections accompanied by a flattened cell morphology. It should be noted that slight differences in spreading were observed by confocal microscopy of the actin cytoskeleton (Fig. 2C) versus phase-contrast microscopy (Fig. 5B), which can be attributed to the methodological differences between these techniques.

Taken together, these data point to a dominant cell-binding region common to N18 and 17–27. Their mapped shared sequence is domains 17–18, which are distinct from the EBP-binding region (domain 24 (14)), the GAG-binding site (C terminus (11)), or the RKRK/integrin αvβ3-binding site (domain 36 (12)).

HDF Attachment to the Central Region of Tropoelastin Is Independent of EBP and HS—Lactose inhibits EBP-dependent cell binding to tropoelastin (21). Furthermore, HS blocks GAG-mediated cell binding (11). To determine whether the central region utilizes EBP or a GAG-mediated mechanism, cell adhesion was performed in the presence of α-lactose, β-lactose, the control d-glucose, or HS (data not shown). α-Lactose, β-lactose, and d-glucose did not perturb cell attachment to WT, ΔRKKR, or N18. Also, HS did not significantly affect HDF attachment. These data reveal that HDF adhesion to this region is not driven primarily by either of these mechanisms.
HDF Attachment and Spreading on N18 and 17–27 Is Mediated via αV-containing Integrin(s)—Integrins are inhibited by chelation of cations, so we examined the effect of EDTA on cell binding to ΔRKRK, N18, and 17–27 (Fig. 6A). In the presence of 5 mM EDTA, cell attachment to WT, ΔRKRK, N18, and 17–27 decreased substantially, suggesting an integrin-mediated cell-binding mechanism. Concentration gradients of Ca\(^{2+}\), Mg\(^{2+}\), and Mn\(^{2+}\) were added back to HDFs in cation-free buffer (Fig. 6B). Consistent with EDTA inhibition, only background levels of cell attachment were seen in cation-free buffer for WT, ΔRKRK, N18, and 17–27. Cell attachment was stimulated on all four tropoelastin constructs using up to 0.4 mM Mn\(^{2+}\) -containing buffer in a dose-dependent manner. Mg\(^{2+}\) also stimulated cell attachment but at lower levels compared with Mn\(^{2+}\). Ca\(^{2+}\) stimulated low levels of cell attachment at all concentrations.

This pattern of cation dependence is characteristic of integrin-mediated cell interactions (26). Therefore, to determine the specific integrin subunits involved, a panel of inhibitory anti-integrin antibodies were included during cell spreading studies to inhibit defined integrin subunit function (Fig. 7A). Cell spreading assays were performed in preference to cell attachment assays because subtle differences in cell interactions are more noticeable in cell spreading assays due to the potential for changes in cell morphology that would not necessarily result in altered cell attachment (27). Inclusion of the pan-anti-α\(^v\_) integrin inhibitory antibody 17E6 significantly inhibited cell spreading on WT (26.2 ± 7.3%), ΔRKRK (11.1 ± 3.9%), N18 (2.2 ±
3.7%), and 17–27 (0 ± 0%) compared with no-antibody control cell spreading (80.9 ± 5.4%, 73.4 ± 5.9%, 42.1 ± 6.5%, and 27.7 ± 13.7%, respectively). In contrast, 17E6 did not significantly alter cell spreading on negative control BSA-coated wells (data not shown). 17E6 inhibition can be explained by the need for an αv-containing integrin for cell attachment to domains 17 and 18 of tropoelastin. Neither P1D6 (anti-α5β1) nor the non-immune IgG control inhibited cell spreading on the constructs. To test the functionality of P1D6, spreading of HDFs on an α5β1 ligand (fibronectin) in the presence of 10 and 20 μg/ml P1D6 was compared with that on N18 (Fig. 7B). Inclusion of P1D6 at both concentrations significantly inhibited cell spreading on fibronectin (69.0 ± 19.2% and 58.3 ± 9.2%, respectively) compared with the no-antibody control (97.0 ± 2.9%); however, cell spreading on N18 remained unchanged. Integrin αvβ3 binding is specific for the C terminus of tropoelastin (12), so we tested for other αv-containing integrin binding. P1F6 (anti-αvβ3) inhibited cell attachment on N18 (21.9 ± 0.4%) and 17–27 (11.7 ± 3.7%); this contrasted with the no-antibody control (44.1 ± 6.8% and 29.6 ± 6.8%, respectively). There was no P1F6-dependent inhibition of cell attachment to WT (78.1 ± 3.8%) and ΔRKRK (67.9 ± 3.9%) compared with the no-anti-
body control (78.6 ± 4.1% and 73.9 ± 7.8%, respectively) (Fig. 8A). In contrast, inclusion of P1F6 inhibited cell spreading on WT (60.1 ± 4.2%), ΔRKRK (31.5 ± 8.8%), N18 (8.2 ± 4%), and 17–27 (10.1 ± 7.2%) compared with no-antibody control cell spreading (80.9 ± 5.4%, 73.4 ± 5.9%, 42.1 ± 6.5%, and 27.7 ± 13.7%, respectively) (Fig. 8B). To control for P1F6 specificity, HDF spreading on a non-αvβ3 ligand (collagen type I) in the presence of P1F6 was compared with that on tropoelastin constructs N18 and 17–27 (Fig. 8C). As shown previously, inclusion of P1F6 inhibited cell spreading on N18 (10.7 ± 1.9%) and 17–27 (4.4 ± 2.7%) compared with the no-antibody control (60.2 ± 8.3% and 50.9 ± 10.3%, respectively). In contrast, no inhibition of cell spreading on collagen type I was observed. These data reveal that the αv-containing integrin αvβ3 interacts with the dominant cell-binding region common to tropoelastin constructs N18 and 17–27 (Fig. 9).

**DISCUSSION**

Previous studies investigating the binding of fibroblasts to tropoelastin have shown that integrin αvβ3 mediates cell binding to the C-terminal GRKRK sequence (12). Persistent cell binding to a ΔRKRK construct suggests the existence of another RKRK-independent cell-binding site(s) within tropoelastin. This is consistent with previous studies, as peptide inhibition of RKRK-dependent cell binding was not capable of fully blocking cell attachment to tropoelastin (12). Instead, WT and ΔRKRK supported a noticeable difference in the actin fiber assembly. Actin fiber assembly requires appropriate cues from the surrounding ECM. Therefore, the RKRK motif may have a significant role in cell signaling. Alternatively, it is plausible that the RKRK motif is one of a number of cell adhesion sites present on tropoelastin and that ligation of one site by a cell is sufficiently
strong enough for cell adhesion but insufficiently secure for cytoskeletal assembly. As such, full cell binding to tropoelastin is an additive process requiring simultaneous binding by multiple sites.

To locate the RKRK-independent binding regions, we examined further C-terminal truncations of tropoelastin to identify potential new cell adhesion sites. N25, N18, N10, and 17–27 supported cell binding. Although N10 could support cell binding, it was at a much lower level compared with the other constructs, so N10 was not considered sufficiently cell-adhesive to be responsible for RKRK-independent cell attachment. Inclusion of domains 19–25 in N25 over those present in N18 did not influence cell binding, so domains C-terminal of domain 18 were deemed to be unnecessary for cell adhesion to this new site. 17–27 consistently showed cell binding behavior that was similar to N18, indicating that cell binding occurred in the overlapping domains 17 and/or 18. Subtle differences in cell affinity between N18 and 17–27 were observed that could be accounted for by the presence of domains 2–10 in N18 but not in 17–27. N10 contains a low affinity cell-binding site, so the subtle differences between N18 and 17–27 may simply reflect the additional site contained in domains 2–10 of N18 compared with 17–27. With increasingly stringent washing conditions, the cell-binding affinities for N18 and 17–27 were comparable, which is consistent with the inclusion of a low affinity cell-binding site in N18 that is sensitive to more stringent washing regimes. It could be argued that C-terminal tropoelastin truncations may lead to changes in the three-dimensional folding of these truncated constructs. However, the solution structures of WT, N25, and N18 have all been solved by small angle x-ray scattering, showing that these three constructs possess structures that are superimposable (28). Therefore, it is unlikely that the different cell-binding affinities can be attributed to gross unfolding of the C-terminal truncated tropoelastin constructs. We conclude that the persistent cell binding observed on N18 and 17–27 is due to the existence of a common binding site within domains 17 and 18 of the overlapping region in these constructs.

Cell adhesion is the initial step in cell interactions with ECM proteins, which can engage transmembrane integrins and trigger a signaling cascade resulting in a flattened cell morphology (27). This requirement for both cell engagement and cell signaling allows for differences in cell interactions to be ascertained by cell spreading analysis (29). HDFs were capable of spreading on WT, ΔRKRK, N18, and 17–27. Similar to the attachment data, spreading on N10 was significantly higher than on the background control but lower than on the other tropoelastin constructs. There were no significant differences in cell areas between N18 and 17–27. Additionally, N18 and 17–27 supported significantly larger cell areas than N10 and the background control. This result is consistent with a model in which a site within N18 and 17–27 can attach to and subsequently elicit a spreading response in HDFs.

We sought to identify the cell receptor responsible for mediating this newly identified fibroblast-tropoelastin interaction. Lactose inhibition studies showed that fibroblast attachment to the 17–18 cell-binding site was not mediated by EBP. This lack of involvement of EBP is consistent with several studies showing that full-length tropoelastin cell attachment is EBP-independent (21). GAG-mediated cell binding has been observed between HDFs and the C-terminal 25 amino acids of bovine tropoelastin. This is in contrast with our current results, as HS does not inhibit HDF attachment to human tropoelastin (12, 21). The C termini of human and bovine tropoelastin share a high degree of homology in the domain encoded by exon 36; however, human tropoelastin does not contain domains 34 and 35, which are required in bovine tropoelastin for HS-dependent cell binding. Therefore, our lack of HS-dependent cell attachment is entirely consistent with the assertion that bovine tropoelastin but not human tropoelastin contains a HS-binding site at the C terminus (12).

Integrins are dominant mediators of cell interactions with ECM components (1). They exist in multiple conformations, and it is these conformations that result in switching of integrin activity. This activity can be modulated in vitro by exogenous divalent cations or function-blocking monoclonal antibodies as

![Schematic of the integrin-binding sites on human tropoelastin.](image-url)
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a probes for integrin-mediated interactions (2, 30). EDTA inclusion suggested that cell binding to the 17–18 cell-binding site on tropoelastin may be integrin-mediated. This was supported by the divalent cation dependence of cell interactions that control for nonspecific EDTA effects. The cation dependence exhibited on WT, ΔRKRRK, and N18, where Mn$^{2+}$ stimulated maximal cell binding, Mg$^{2+}$ stimulated intermediate cell binding and Ca$^{2+}$ stimulated low cell binding, is indicative of an integrin-mediated cell-binding interaction (12).

Monoclonal antibody inhibition studies identified α$_{5}$-containing integrin involvement in fibroblast binding to the 17–18 cell-binding region of tropoelastin. Integrin α$_{5}$β$_{3}$ has been shown to mediate the interaction between fibroblasts and the C terminus of tropoelastin, so the possibility of other integrins-tropoelastin interactions was explored. The inclusion of P1F6 (anti-α$_{5}$β$_{3}$) significantly inhibited cell attachment to the N18 and 17–27 tropoelastin constructs. The lack of inhibition observed for WT and ΔRKRRK could be due to other mechanisms that potentially compensate for α$_{5}$β$_{3}$ blockade. To investigate this further, P1F6 was included in cell spreading assays, showing partial inhibition of WT- and ΔRKRRK-dependent cell spreading. Inhibition of cell spreading on 17–18-containing constructs identified that integrin α$_{5}$β$_{3}$ is involved in mediating fibroblast interaction with this new cell-binding site. The utilization of this integrin for cell-tropoelastin interaction is consistent with integrin α$_{5}$β$_{3}$ expression on fibroblasts (5, 31), where it plays a role in cell adhesion to ECM proteins, including vitronectin and fibronectin (3, 31, 32). Furthermore, this binding region overlaps with part of the molecule that is implicated in tropoelastin self-association and elastic fiber formation (33).

In this work, we have determined that integrin α$_{5}$β$_{3}$ can interact with tropoelastin through domains 17 and 18, in addition to the previously published interaction of integrin α$_{5}$β$_{3}$ with the C-terminal RKRRK motif. This does not exclude the possibility that other integrins may be involved in mediating cell adhesion to this region.

Integrins, including α$_{5}$-containing integrins, are involved in cell adhesion, migration, and invasion of tumor cells and play an important role in neoangiogenesis and tumor cell proliferation. Abnormal expression of integrin α$_{5}$β$_{3}$ has been implicated in the pathogenesis of various diseases. More specifically, fibroblasts in the autoimmune disease scleroderma have increased expression levels of integrin α$_{5}$β$_{3}$ (5). In additional, integrin α$_{5}$β$_{3}$ promotes angiogenesis via a pathway that is distinct from integrin α$_{5}$β$_{3}$-dependent angiogenesis. Integrin α$_{5}$β$_{3}$ is also essential for the differentiation of endothelial progenitors to differentiated endothelial cells (34, 35). Therefore, further investigation of integrin α$_{5}$β$_{3}$-tropoelastin interactions could lead us to a better understanding of these disease and physiological states. In summary, in this study, we investigated the potential for additional RKRRK-independent cell-binding sites in human tropoelastin. We found that although RKRRK is required for the full cell-interactive properties of human tropoelastin, it is not the sole cell-binding region. Instead, we found that cells could adhere to a central section of tropoelastin encompassing domains 17 and 18 via integrin α$_{5}$β$_{3}$ on the cell. This understanding of the cell adhesion activity of tropoelastin gives improved insight into the physiological and pathological cell responses to this protein.

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