Cell-type Specific Recognition of RGD- and Non-RGD-containing Cell Binding Domains in Fibrillin-1*

(Received for publication, September 21, 1995, and in revised form, November 24, 1995)

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The fibrillins are large glycoprotein components of 10-nm microfibrils found in the extracellular matrix of most tissues. Microfibrils play a role in elastic fiber assembly and serve to link cells to elastic fibers in the extracellular matrix. To determine whether fibrillin-1 specifically interacts with receptors on cells from fibrillin-rich tissues, we evaluated whether two cell types that produce different types of fibrillin can adhere to purified fibrillin-1 in cell adhesion assays. Our results indicate that both cell types attach and spread on fibrillin-1 and that the RGD sequence in the fourth 8-cysteine motif mediates this interaction. Fibroblast attachment to fibrillin-1 was sensitive to inhibition by antibodies to the αvβ3 receptor and by peptides encoding the RGD sequence in fibrillin-1 and the second RGD sequence in fibrillin-2. In contrast, adhesion of auricular chondroblasts to fibrillin-1 was only partially inhibited by these reagents, suggesting that some cell types recognize a second, non-RGD binding site within the fibrillin molecule. These findings confirm and extend ultrastructural studies that suggest a direct interaction between microfibrils and the cell surface and provide a functional explanation for how this association occurs.

Microfibrils are filamentous structures with a diameter of 10–12 nm found in the extracellular matrix of most tissues. Although usually associated with elastic fibers, microfibrils devoid of elastin are also found in abundance in tissues that are subject to mechanical stress where they are thought to provide a link between the cell and underlying elastic structures (1). The term microfibril was first used by Low (2) as an arbitrary morphological descriptive term for all filaments in the extracellular space with a diameter of less than 20 nm lacking a typical collagen banding pattern. Recent studies have proven this definition to be too broad since some filaments that fit Low's description are, in fact, collagenous in nature or react with antibodies to known matrix molecules such as fibronectin or proteoglycans. It is thus apparent that many matrix components may exist in the form of microfibrils and that morphology alone cannot be used to characterize these structures (3).

Within the past few years, the direct biochemical and immunological characterization of microfibrillar components has led to a more accurate definition of a microfibril. Several proteins, including AMP, microfibril-associated glycoprotein (MAGP), fibrillin-1, and fibrillin-2, have been shown to be major components of these structures (4–7), although other proteins are associated with microfibrils in some tissues (4, 8). While our understanding of the composition of these unique filaments is still emerging, immunological studies suggest that fibrillin is a common constituent of all microfibrils, and hence, the presence of this protein provides a minimal definition of what can be called a microfibril. Even so, this definition includes an element of complexity since differences in the spatial and temporal expression of fibrillin-1 and fibrillin-2 (6, 9) implies that microfibrils may consist of one protein or the other, or both. When associated with elastic fibers, microfibrils are thought to provide the basic scaffolding for elastic fiber organization. The initial stages of assembly occur at regions of the cell surface where microfibrils frequently appear attached to the plasma membrane. Ultrastructurally, these areas of attachment resemble focal contacts with an abundance of microfilaments and cytoskeletal elements on the cytoplasmic side of the membrane (10). An association with membrane-associated dense plaques has also been observed for microfibrils that serve to anchor endothelial and epithelial cells to underlying elastic fibers. The propensity of cytoskeletal elements to cluster where microfibrils contact the cell surface suggest that protein components of the microfibril interact with cell-surface receptors which, in turn, serve as a transmembrane link between microfibrils and components of the cytoskeleton.

Of the known microfibrillar proteins, the fibrillins are the best candidates for interacting with cell surface receptors. The two known fibrillin gene products manifest the same general motif structure, but functional differences between the proteins are suggested by a disparity in their pattern of expression (6) as well as by the distinct clinical phenotypes resulting from mutations in one or the other genes (11). Structural similarities in fibrillin-1 and fibrillin-2 consist of clusters of epidermal growth factor-like domains separated by 8-cysteine domains that were first described in transforming growth factor-β1-binding protein (11, 12). Overall, the amino acid homology between the two fibrillins approaches 70%, and all but one of the putative N-linked glycosylation sites are conserved. The most striking

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* This work was supported by National Institutes of Health Grants HL-26499 and HL-41926 (to R. P. M.), AR-41474 (to J. R.), CA50286 and CA45726 (to D. A. C.), and AR42044 (to F. R.). This article is 165 from the Brookdale Center for Molecular Biology. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: MAGP, microfibril-associated glycoprotein; FCL, fetal bovine ligamentum nuchae; FBC, fetal bovine auricular cartilage; LTBP-2, latent transforming growth factor-β-binding protein-2; DMEM, Dulbecco’s modified Eagle’s medium; BSA, bovine serum albumin.
sequence divergence is found at the amino terminus (Fig. 1, domain A, 19% homology) and at the end of the first 8-cysteine motif where a proline rich sequence in fibrillin-1 is replaced with a glycine rich sequence in fibrillin-2.

The possibility that fibrillin might interact with cell receptors is suggested by the presence of RGD sequences in both fibrillin molecules. Fibrillin-1 contains one RGD sequence located in the fourth 8 cysteine motif whereas the RGD in the fourth 8-cysteine motif of fibrillin-1 is replaced by a proline rich sequence in fibrillin-2. Fibrillin-2, in contrast, has two RGD sequences that are located in the second in the third 8-cysteine motif where a proline rich sequence in fibrillin-1 is replaced with a glycine rich sequence in fibrillin-2.

In this study we evaluated whether fibrillin specifically interacts with receptors or binding proteins on cells found in fibrillin-rich tissues. Using adhesion assays, we have shown that cells specifically interact with fibrillin-1 and that the RGD-containing domain defines a major cell binding epitope. The integrin αvβ3 is the adhesion receptor responsible for binding, although other integrins may interact with this sequence under some conditions. Our data also suggest a second, non-RGD receptor-binding site in fibrillin, that is recognized by some cell types.

**EXPERIMENTAL PROCEDURES**

**Materials—** Ellman’s reagent, ImmunoPure Sulfo-NHS-Biotinylation kit, IODOGEN, and m-maleimidobenzoyl-N-hydroxysuccinimide ester were purchased from Pierce. Plates used for adhesion assays were from Costar. Antibodies to fibrillin-1 and fibrillin-2 were raised against synthetic peptides (see below) encoding the RGD sequences of these proteins (9). Rabbit polyclonal antibodies to αv, α3, α5, and β1 integrin subunits, and a rat monoclonal to the β3 subunit were generously provided by Dr. John McDonald. Polyclonal antibodies to fibronectin, vitronectin, and to the α5β1 integrin were from Telios Pharmaceuticals. Antibodies to αv (LM142) and αvβ3 (LM609) have been described previously (13). All other reagents were from Sigma.

**Synthetic Peptides and Antibodies—** Peptides to the RGD sequences in human fibrillin-1 and fibrillin-2 were synthesized using an ABI model 431A synthesizer employing t-butoxycarbonyl chemistry. The sequence of each peptide was confirmed by sequence analysis using an ABI 473 protein sequencer. Peptide Fib-1d-RGD (YLDPRPGQGNDTA) includes the RGD sequence of fibrillin-1, peptide Fib-2-RGD1 (AGFANRGDVLGT) contains the first RGD sequence of fibrillin-2 located in the third 8-cysteine domain, and peptide Fib-2-RGD2 (YLKFGRPGDGSL) contains the second RGD sequence in fibrillin-2. The locations of RGD sequences in fibrillins-1 and fibrillin-2 are shown schematically in Fig. 1. Control peptides included Fib-1d-RGQ (Gln substituted for Asp, YLDPRPGQGNDTA) and GRGDPS. Antibodies to the fibrillin peptides have been described previously (9). By immunoblot analysis, the Fib-1 peptide antibody did not react with fibrillin-2 or fibronectin. Conversely, the antibody to the Fib-2 peptide reacted with fibrillin-2 but not fibrillin-1. Antibodies to fibronectin did not react with either fibrillin-1 or fibrillin-2.

Fab fragments of the Fib-1d-RGD antibody were generated by papain digestion and reductive alkylation. IgG purified from the immune serum was dialyzed into 70 mM sodium acetate, 50 mM NaCl, pH 4.0, concentrated to 5 mg/ml, and treated with papain overnight at 37°C at a 1:33 enzyme to substrate ratio. Following buffer exchange into 0.5 M Tris, pH 8.3, the digest was reduced with 10 mM 2-mercaptoethanol for 1 h at room temperature. Iodoacetamide was added to 12 mM, and the digest was incubated for 15 min on ice in the dark. The Fab fragments were then dialyzed into 7.5 M Tris, 150 mM NaCl, pH 7.5, and purified on a 1.5 x 90 cm Sephacryl S-200 column equilibrated in the same buffer.

**Cdls—** Fibrilons from fetal bovine ligamentum nuchae (FCL cells) and chorondoblasts from fetal bovine auricular cartilage (FBC cells) were cultured as described elsewhere (14). Cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% calf serum and were used before passage 3.

**Fibrillin Purification and Radiolabeling—** Microfibrillar proteins were isolated from fetal bovine ligamentum nuchae using a modification of the reductive saline procedure of Gibson et al. (4). Proteins in the 6–10-kD range were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electroblotted to nitrocellulose. The nitrocellulose was probed with rabbit polyclonal antibodies to fibrillin, followed by mouse monoclonal antibodies to fibrillin and horseradish peroxidase conjugated antibodies and developed with a chemiluminescent substrate. The fibrillin bands were excised from the gel and suspended in 6 M guanidine and 50 mM HEPES, pH 7.4, 150 mM NaCl, 0.1% Nonidet P-40. The fibrillin peptides have been described previously (9). By immunoblot analysis after alkylation as described by Gibson et al. (4), fibrillin was radiolabeled by using approximately 30 μg of purified protein with 300 μCi of Na125I in IODOGEN-coated glass tubes for 10 min at room temperature. Labeled protein was separated from free iodine by gel filtration using Sephadex G25.

**Cell Attachment Assay—** Polystyrene 96-well plates were incubated at room temperature overnight with fibrillin-1 diluted in Tris-buffered saline or bovine serum albumin (BSA) conjugated with synthetic peptides in the same buffer. Unbound sites were then blocked with 1 mg/ml BSA in phosphate-buffered saline. Using 125I-labeled fibrillin to evaluate coating efficiency, we found that approximately 90% of the added protein up to a concentration of 30 μg well bound to the plate. The optimal concentration of fibrillin that supported cell adhesion was determined by coating wells with fibrillin ranging from 0–200 μg/ml. First to third passage cells were harvested by trypsinization, washed once in complete DMEM supplemented with 10% calf serum and twice in serum-free DMEM containing 1 mg/ml BSA. Cells were resuspended in 200-μl aliquots of binding buffer (DMEM containing 1 mg/ml BSA) were added to each well and incubated at 37°C for periods of up to 2 h. Nonadherent cells were removed gently in triplicate. The number of adherent cells was quantified using the hexosaminidase assay of Landegren (15). Maximal cell attachment was achieved at concentrations of 10 ng of fibrillin-1/well, which was the concentration used for all adhesion studies.

**Peptide competitive adhesion assay—** Wells were added to the same cells as the above ones. In experiments using antibodies, the cell suspension and the fibrillin-containing well were preincubated for 30 min at room temperature with antibodies dissolved in binding buffer. Adhesion was then carried out in the usual manner.

**Fig. 1.** Domain map of fibrillin-1 and fibrillin-2. Letters at the top indicate the five structurally distinct regions of the molecule. The repeating structural elements are defined at the bottom of the figure. The location of RGD sequences are shown along with the synthetic peptides that were used for adhesion assays and antibody production.
Immunofluorescence Analysis of Receptor Localization—Adherent cells were suspended by a 10-min treatment with Versene, washed, and allowed to adhere to multichamber slides coated with fibrillin-1 in the absence of serum for 1 h at 37 °C. The nonadherent cells were then removed, and the adherent cells were fixed with 3% paraformaldehyde in Hanks’ balanced salt solution for 30 min. The fixed cells were permeabilized with 0.5% Triton X-100 for 1 min, washed, and blocked with 5% BSA in phosphate-buffered saline. The permeabilized cells were stained with antibody to the β3 integrin subunit for 60 min at room temperature, washed, and incubated with fluorescein isothiocyanate-conjugated goat anti-rat IgG. After washing, the slides were analyzed by fluorescence microscopy.

RESULTS

Purification of Bovine Fibrillin-1—Microfibrillar proteins were extracted from fetal bovine ligamentum nuchae using a modification of the reductive saline procedure of Gibson et al. (4). This approach utilizes repeated extraction with high salt and guanidine to remove soluble proteins before microfibrillar components are solubilized with a final extraction incorporating reducing agent. The ligamentum nuchae was chosen for this study because it is highly enriched in microfibrils containing fibrillin-1 with only low levels of fibrillin-2 (9). Throughout the isolation procedure, extraction supernatants and column fractions were monitored by immunoblot analysis for the presence of fibrillin-1, fibrillin-2, MAGP, fibronectin, vitronectin, and LTBP-2. This latter protein, which has many properties in common with fibrillin (16), has been shown to be an integral component of microfibrils and a potential contaminant of fibrillin preparations (17).

Dot blot immunoassay of sequential extraction supernatants showed that fibronectin was present at all stages of the purification, although only small amounts of the protein were detected in the final extract. Vitronectin, in contrast, was found in the first pellet wash and was not found in later extractions. Gibson et al. (4) have shown that the final reductive extract is highly enriched in microfibrillar components and contains essentially five proteins: fibrillin, LTBP-2, MAGP, fibronectin, and MP25. When this extract was fractionated over a CL-4B column, fibrillin-1 was found to elute as a broad peak shortly after the void volume. On SDS-polyacrylamide gel electrophoresis, the leading fractions in this peak were shown to contain a single protein at ~340 kDa that reacted with the Fib-1 antibody. This protein did not react with antibodies specific for fibrillin-2, fibronectin, or vitronectin, nor were fibronectin or vitronectin detected in these fractions by Western blot (not shown). Confirmation that the 340-kDa protein was fibrillin-1 was obtained by partial sequence analysis of a cyano gen bromide peptide generated from the parent protein (data not shown). Later fractions in this peak contained several lower molecular weight bands that reacted with the Fib-1 antibody and were presumed to be degradation products of fibrillin-1. Fibronectin and LTBP-2 eluted in fractions that overlapped with the receding end of the fibrillin-1 peak (Table I). Smaller microfibrillar components, including MP78/70 and MAGP (4), eluted later in the column run.

To increase our yield of purified fibrillin, fibrillin-1-containing fractions from the CL-4B column were pooled as indicated in Table I and were fractionated using DEAE followed by heparin-Sepharose. Immunoblot analysis showed that DEAE effectively separated LTBP-2 from fibrillin-1 and a small amount of fibronectin, but the later two proteins eluted as overlapping peaks which could be separated using heparin-Sepharose. Fig. 2 is a SDS-polyacrylamide gel electrophoresis analysis of purified fibrillin-1 showing a single band at the expected molecular mass of approximately 340 kDa. Western blot analysis confirmed the absence of fibronectin in the final product.

Cell Attachment to Fibrillin-1—To study possible interac-

![Image](357x234 to 513x391)

**Fig. 2. Characterization of purified fibrillin-1.** Lane A shows Coomassie Blue staining of a 5% SDS-polyacrylamide gel containing fibrillin-1 purified from fetal bovine ligamentum nuchae (pooled fractions 29–33 from heparin-Sepharose chromatography, see Table I). Lanes B and C are immunoblots transferred from 10% gels developed with an antibody to fibronectin and lanes D and E are developed with the fibrillin-1d antibody. Lanes B and D contain a commercial preparation of fibronectin and lanes C and E contain the fibrillin preparation shown in lane A. Molecular weight standards are × 10^6.
Inhibition of Cell Attachment by RGD-containing Peptides from Fibrillin-1—Specific cell binding sites within the fibrillin molecules have been suggested by the presence of RGD sequences (see Fig. 1). To determine whether these sites mediate attachment and spreading of FCL and FBC cells, synthetic peptides encoding these sequences were added together with cells to fibrillin-1-coated wells. As shown in Fig. 4A, the number of FBC cells showing attachment to fibrillin-1 was reduced in a concentration-dependent fashion by the Fib-1d-RGD and GRGDSP peptides. However, maximal inhibition by Fib-1d-RGD peptide was never greater than 50%, even at extremely high peptide concentrations. Synthetic GRGDSP peptide was a slightly better inhibitor than the fibrillin-1 sequence but even at the highest concentration tested, GRGDSP was only able to inhibit binding by 65%. Attachment of FCL cells, in contrast, was completely inhibited by GRGDSP and was inhibited 70% by Fib-1d-RGD peptide (Fig. 4B). Peptide Fib-2-RGD2, encoding the RGD sequence in fibrillin-2 located in the same 8-cysteine domain as fibrillin-1, also inhibited adhesion of FBC cells to purified fibrillin-1, although to a lesser extent (Fig. 5). Peptide Fib-2-RGD1 was ineffective at blocking adhesion of either cell type, as was the control peptide, Fib-1d-RGQ. It is important to note that FBC cells that remained attached to fibrillin-1 in the presence of Fib-1d-RGD and GRGDSP peptides had a rounded morphology and did not spread during the 2-h assay period (Fig. 6).

The ability of the fibrillin-1 RGD domain to support adhesion was tested directly by adding Fab fragments of affinity purified Fib-1d-RGD antibody to the adhesion assay. Fig. 7 shows that FCL cell attachment to fibrillin-1 was inhibited by 70% in the presence of 100 μg/ml of this antibody, FAB fragments of an antibody to another microfibrillar component, MAGP, had no effect on adhesion of FCL cells and served as a negative control.

Antibodies Directed to αvβ3 Integrin Block FCL and FBC Adhesion to Fibrillin-1—Because affinity chromatography and peptide blocking studies were strongly suggestive that αvβ3 is a major adhesion receptor for fibrillin-1, we examined whether antibodies to the αvβ3 receptor complex could inhibit binding of FCL and FBC cells to fibrillin-coated dishes. Fig. 8 shows the results of an adhesion assay where cells were preincubated with an antibody to the αvβ3 complex. This antibody was able to block binding of FBC cells by ~60%, which was similar to the inhibition of attachment observed with the Fib-1d-RGD and GRGDSP peptides. An antibody directed against the αv subunit alone did not prevent cell attachment and was used as a negative control. Interestingly, an antibody to the fibronectin receptor (α5β1) stimulated cell adhesion (Fig. 8).

Integrin αvβ3 Localizes to Focal Contacts in Cells Attached to Fibrillin-1—As shown in Fig. 9, FCL cells which spread on purified fibrillin-1 express β3 in focal contacts, indicating that αvβ3 expression promotes the assembly of adhesion plaques and organization of the cytoskeleton leading to cell spreading. The β3 staining pattern of cells plated on fibrillin is identical to the distribution of focal contacts formed when these cells attach to plates coated with serum.

**DISCUSSION**

The objective of this study was to determine whether fibrillin-1 could specifically interact with binding proteins on the
surface of two phenotypically distinct cell types that reside in fibrillin-rich tissues: (a) fibroblasts from the ligamentum nuchae (FCL cells) and (b) chondroblasts from auricular cartilage (FBC cells). Both cell types are specialized to synthesize large amounts of fibrillin, but differ in the fibrillin types they produce, with fibrillin-1 being the major form secreted by FCL cells and fibrillin-2 by FBC cells (9). Thus, it was of great interest to determine whether these two cell types demonstrate adhesive properties that correlate with their differing fibrillin phenotypes.

When tested in adhesion assays, we found that purified fibrillin-1 was an adhesive protein for both cell types and that the RGD-containing domain defined a major cell binding epitope. The importance of the RGD domain for cell interactions with fibrillin was established using soluble peptides as competitive inhibitors and by comparing cell attachment and spreading on the intact, purified protein. Our results suggest that the RGD sequence in the fourth 8-cysteine motif in both fibrillin-1 and fibrillin-2 is active in cell attachment, and is most likely recognized by the \( \alpha v \beta 3 \) receptor. The RGD sequence in the third 8-cysteine domain of fibrillin-2 may be either inactive or specific for a different receptor. The inability of this sequence to block adhesion to fibrillin demonstrates an element of specificity in the interaction and suggests that inhibition is not a general property of any RGD peptide.

As with many other matrix proteins, cell binding to fibrillin appears to be cell-type specific. This was suggested by studies showing that attachment of FBC cells to intact fibrillin-1 could only be partially inhibited with RGD peptides or receptor antibodies. In contrast, adhesion of FCL cells was completely inhibited by RGD-containing peptides. These results suggest that FCL fibroblasts mainly recognize the RGD sequence, whereas FBC cells recognize, in addition to the RGD sequence,
a second non-RGD site somewhere in the fibrillin-1 molecule. The nature of the second binding site on fibrillin-1 is unknown, although cells that attach to fibrillin in the presence of RGD peptide fail to spread, suggesting that the receptor that binds to this site does not interact with the cytoskeleton in a way that promotes spreading.

An interesting and surprising result from our antibody inhibition studies was enhanced FBC cell binding to fibrillin in the presence of an antibody to α5β1 integrin. We interpret this result to suggest that the antibody induces a change in either α5β1 receptor affinity or specificity, thereby converting the receptor to a form that can bind fibrillin. This is similar to the finding by Faull et al. (18) that a β1 antibody stimulates adhesion of erythroleukemic cells by converting the fibronectin receptor from a low to a high affinity state. Other examples of cell surface activation of integrin receptors have been described elsewhere (19).

Our identification of cell binding activity in fibrillin-1 purified from tissues with reductive guanidine raises the question of whether domains responsible for cell adhesion are functional in the native molecule or exist as cryptic sites that are activated upon protein denaturaton. While the inability to extract and purify native fibrillin-1 from normal tissues currently precludes an experimental evaluation of this question, other laboratories have obtained preliminary data suggesting that the native molecule does specifically interact with cells. For example, Withers et al. (20) demonstrated attachment and spreading of fibroblasts to a bacterially expressed fusion protein containing a fragment of fibrillin-1 containing the RGD sequence. Similarly, Kielty et al. (21) found that smooth muscle cells attach and spread on intact fibrillin-containing microfibrils purified from fetal bovine skin. Although both studies document interactions between cells and fibrillin, neither study identified the receptor responsible for this binding.

The biological role of receptors for fibrillin is undoubtedly complex. Morphological studies have documented extensive interactions between cells and microfibrillar structures in the extracellular matrix. In many instances, these microfibrils form a continuous link between the cell and the extracellular matrix, suggesting that microfibrils not only facilitate elastic fiber assembly but also play a role in cell anchorage to extracellular matrix components. In skin, for example, microfibrils anchor epidermal cells to elastic fibers in the dermis (22) and similar "anchoring filaments" have been identified in the subendothelial matrix of lymphatic capillaries, coronary vessels, and the aorta where they connect endothelial cells to surrounding elastic fibers (23–25). Recently, Davis (1) has shown that these connecting filaments contain fibrillin, confirming their identification as microfibrils. Another interesting property of the connecting filaments is that they are anchored at the cell surface in regions of the membrane occupied on the intracellular face by membrane-associated dense plaques. This is consistent with αvβ3 being a receptor for fibrillin since this integrin is known to associate into focal contacts and to organize the cytoskeleton (26).

In addition to anchoring endothelial and epithelial cells to underlying elastic fibers, fibrillin may also facilitate adhesion or migration of stromal or inflammatory cells. The distribution of fibrillin-1 and fibrillin-2 in developing tissues suggests that both proteins are usually, but not exclusively, associated with elastic fibers (6). In instances where there is no elastin, fibrillin by itself or in association with other microfibrillar components may act as a classical adhesion protein. Alternatively, at sites of inflammation or active tissue remodeling, elastases could strip elastin from microfibrils, thereby exposing fibrillin molecules that are otherwise masked. An invading inflammatory cell with the appropriate receptors could then use fibrillin to move through an elastin-rich tissue such as a blood vessel. Although the present study focused on interactions between fibrillin and normal, elastin-producing cells, other studies in our laboratory have shown that fibrillin is an adhesive protein for cells that do not make elastin, including several tumor cell lines.2

In summary, we have found that fibrillin is capable of specifically interacting with receptors on the cell surface and that the integrin αvβ3 is a receptor for both proteins. This integrin supports cell attachment as well as spreading, suggesting a distinct interaction with the cellular cytoskeleton. Organization of the cytoskeleton at microfibril attachment sites is critically important for elastic fiber assembly and for cell anchorage to surrounding matrix. Our findings suggest a functional explanation for the many ultrastructural studies documenting

the importance of interactions between cells and microfibrils in maintaining tissue integrity.

Acknowledgments—We thank Dr. Elaine C. Davis for helpful discussion and Terese Hall, Clarina Tisdale, and Benjamin R. Mecham for secretarial and technical assistance.

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